Population structures and interaction between the aggressive and non-aggressive subgroups of Ophiostoma ulmi

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POPULATION STRUCTURES AND INTERACTION BETWEEN THE AGGRESSIVE AND NON-AGGRESSIVE SUBGROUPS OF *OPHIOSTOMA ULMI*

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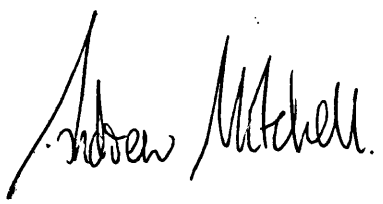

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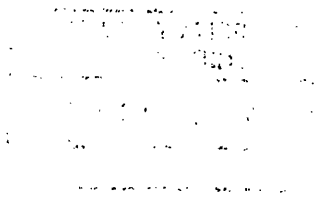


CONTENTS

SUMMARY

Investigation of the saprotrophic phase of the Dutch elm disease pathogen *Ophiostoma ulmi* at sites with pure populations of either the NAN or non-aggressive subgroups of the fungus revealed basic similarities in their population structures. The existence of a mosaic of different genotypes in bark was confirmed for the NAN aggressive and demonstrated for the non-aggressive by using the fungus' vegetative compatibility system as a marker.

Detailed investigation of the NAN aggressive provided further strong evidence for a dynamic, highly competitive saprotrophic phase. Several genotypes were found to establish in each breeding gallery, leading to the mosaic of genotypes in bark, the colonisation of pupal chambers by more than one genotype, and to most beetles carrying spores of more than one genotype.

Pathogenic phase populations of the non-aggressive subgroup in Spain were found to be genetically diverse, with most isolates of a different vegetative compatibilty type. In contrast, a pathogenic phase sample from North America comprised mostly a single vegetative compatibility type. By analogy with populations of the NAN aggressive, this can be explained as the effect of selection pressures resulting from sustained epidemic disease levels on the highly susceptible American elm population.

Investigation of the interaction between the NAN aggressive and non-aggressive in bark colonised by vector beetles and in more controlled experiments in culture and in bark, suggested several factors likely to be important in the replacement of the non-aggressive. These factors are; relative growth rate, pathogenicity to the whole tree during the pathogenic phase, pathogenicity towards dying elm bark with residual host resistance, the relative ability to invade other mycelia *via* the 'penetration effect', the enhanced sporulation associated with the 'penetration effect', and relative sexual and asexual fecundity. Based on these factors and other information a model is proposed for the processes involved in the replacement of the non-aggressive by the aggressive.

1 INTRODUCTION

Elm has been used by man in many different ways for several thousand years, as illustrated by considerable archaeological evidence and frequent references in historic literature. Advantage has been taken of elm timber's strong cross-grain and resistance to decay in contact with water for many specialised products, including bows, wheel hubs and water pipes. Foliage has probably been used since Neolithic times to provide fodder for animals (Heybroek, 1966; Richens, 1983). In addition, elm has aquired a cultural significance beyond its everyday importance, perhaps due to the age and closeness of its association with man. When settling in areas without a suitable native elm flora prehistoric peoples probably took elms with them, eventually making elm a dominant landscape tree in many places. More recently elms have been planted in towns and cities throughout the world.

The genus *Ulmus* is widespread in the north temperate regions, represented by about 18 species. However, the nomenclature and taxonomic status of many described species is uncertain, due partly to the small number of features provided by the simple flowers and fruits, and the extremely variable characteristics of leaves on which most descriptions are based. There is little restriction to hybridization and so some populations may be considered as hybrid swarms showing a continuous range of variation, with perhaps some remnants of pure populations at the extremes of geographical distribution (Melville, 1978). In Europe the possibilities for hybridization have been increased by man transporting elms across the continent (Heybroek, 1976; Richens, 1983), and so the situation is especially difficult. Indeed, Richens (1983) considers that the distribution of the various European elm species can be interpreted almost entirely according to man's influence. Four elm species are generally recognised in Europe; wych elm (*U.glabra* Huds.) with a northern and central distribution, smooth leaved elm *(U.carpinifolia Gleditsch)* with a central and southern distribution, English elm *(U.procera Salisb.)* with a distribution virtually restricted to southern England, parts of Spain and Portugal and perhaps southeast France, and European white elm *(U.laevis Pall.)* with an eastern distribution. Many hybrids are also found, perhaps the most important of which is the Dutch elm group, resulting from hybridization of *U.glabra* and *U.carpinifolia*.

U.carpinifolia is particularly variable with many recognised

varieties, most of which have at some time been described as species. Richens (1977, 1980) includes all these varieties together with *U.procera* in *U.minor* Mill., giving varietal status to *U.procera*, *U.carpinifolia* and some others.

The elm flora of England and its relationship to man has been studied in detail by Richens, and brought together in his comprehensive and scholarly monograph to provide a timely record of its history, botany, utilisation and cultural significance (Richens, 1983). He has hypothesised (Richens & Jeffers, 1978) that the various populations of English and smooth leaved elm in England have been introduced from continental Europe through trading links and the migration of people from the late Bronze Age onwards. Only wych elm is considered to be native. This hypothesis is based on detailed comparisons of biometric characters of elm leaves from many different populations, as well as from archaeological findings and the etymology of place names.

The recent epidemics of Dutch elm disease which have devastated the elm populations of much of Europe and parts of North America and southwest Asia, have therefore had a dramatic impact on man's environment and cultural traditions. The scale of the loss of elms is illustrated by the Forestry Commission's surveys of the southern half of England from 1971-8. Greatly increasing numbers of diseased trees were recorded each year, and by 1978 about 11 million elms had been infected or killed out of a total population of 23 million. The results up to 1976 have been summarised by Gibbs (1978a), who predicted that virtually all mature elms in the survey area would have been killed by 1980, with most surviving trees being found in the East Anglian smooth leaved elm population. Indeed, mature elms are now rare in southern England outside of East Anglia and the control areas of Brighton and other south coast towns. In northern England, Wales and Scotland the effects of Dutch elm disease have been less severe.

1.1 HISTORY AND EPIDEMIOLOGY OF DUTCH ELM DISEASE

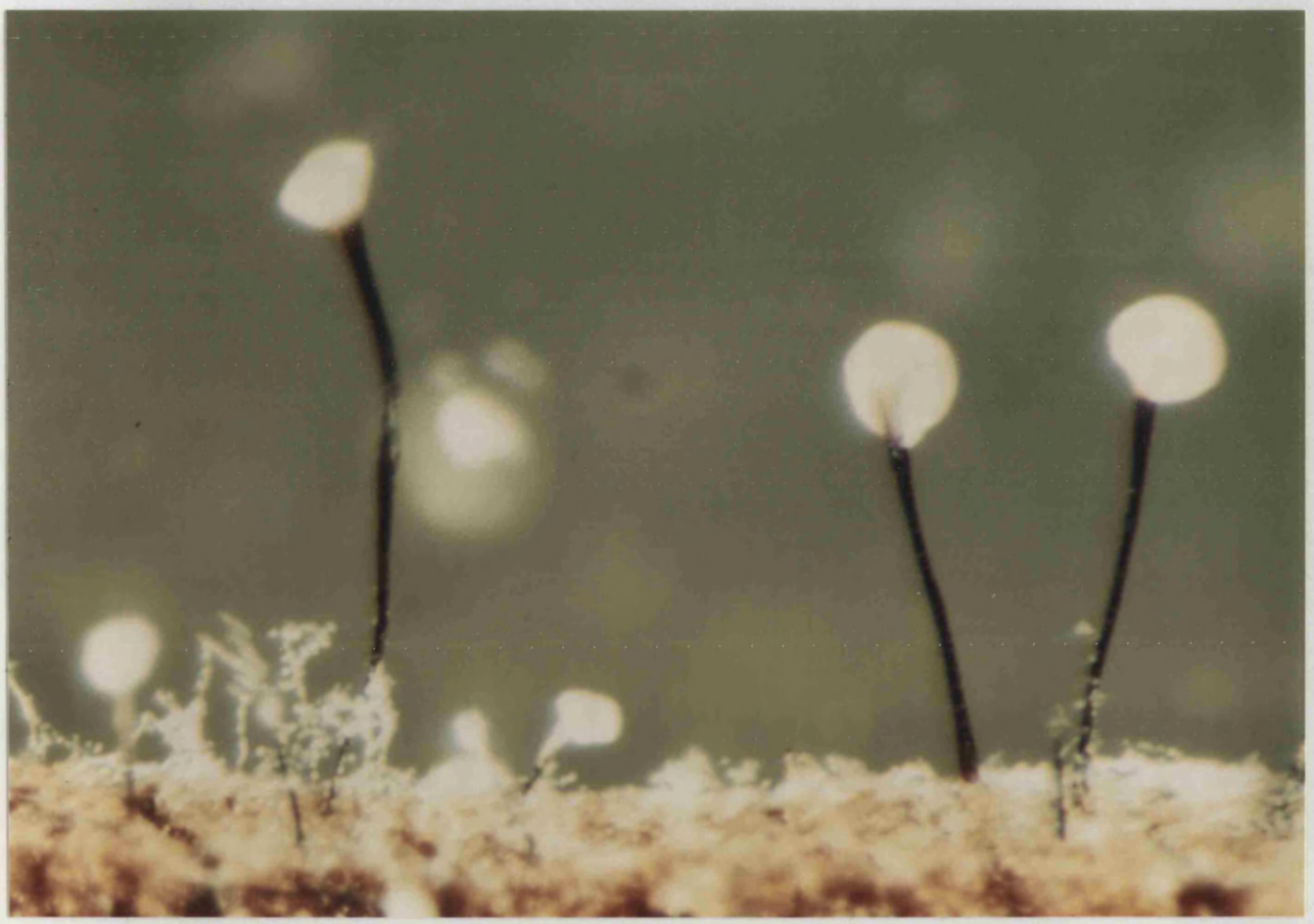
Dutch elm disease was first recorded in Picardy in 1918 (Guyot, 1921), but judging from other reports which soon followed it was probably already widespread in Europe at that time (Peace, 1960). The disease spread rapidly eastwards across Europe, but with a more limited northerly distribution probably due to climatic factors (Gibbs, 1978b). In western Europe this epidemic continued through the 1920s and 1930s, but the disease then gradually declined to endemic levels (Peace, 1960; Heybroek, 1966).

The first epidemic generated considerable interest in the cause of Dutch elm disease, and the disease's name resulted from much early work in the Netherlands. Schwarz (1922) suspected as the cause a fungus which she consistently isolated from the dark vascular staining characteristic of elms with typical wilting symptoms. She named this fungus from the imperfect synnematal stage as *Graphium ulmi*. However, inoculation experiments by Schwarz only produced limited vascular staining, and it was Wollenweber (1928) who conclusively showed G.ulmi to be the causal agent of Dutch elm disease, inducing wilt symptoms in young elms by vascular inoculation.

Four spore stages of the fungus have been recognised, each with an important role at some point in the disease cycle. In addition to the synnematal stage, Schwarz (1922) described the relatively undifferentiated hyaline conidia formed on short sterigmata (the *Sporothrix* stage) (Plate 1.1), and the budding yeast stage. A more detailed description has been given by Hunt (1956), including the sexual perithecial stage first described by Buisman (1932). Although Buisman recognised the heterothallic mating system, designation of the two mating types as A and B derives from the work of Shafer & Liming (1950).

Buisman (1932) renamed *G.ulmi* as *Ceratostomella ulmi* from the sexual stage, but it was placed in the genus *Ophiostoma* by Nannfeldt (Melin & Nannfeldt, 1934), and then in *Ceratocystis* by Moreau (1952). The name *Ceratocystis ulmi* has been in general use since this time, although following reappraisal by de Hoog (1974) and de Hoog & Scheffer (1984) the genus *Ophiostoma* has been separated from *Ceratocystis* on the basis of several important differences. In particular, there is a clear division according to sensitivity to the protein synthesis inhibitor cycloheximide, such that *Ophiostoma* species are unaffected by its presence, but *Ceratocystis* species are

Plate 1.1 O.ulmi Mycelium and Synnemata on Elm Bark



strongly inhibited (Harrington, 1981). This has given the name *Ophiostoma ulmi* (Buism.) Nannfeldt to the causal agent of Dutch elm disease.

There was considerable speculation by earlier workers over the mode of transmission of Dutch elm disease, and although some reports noted scolytid beetles breeding in the bark of dying elms they were considered to be secondary (Spierenberg, 1921). Marchal (1927) was the first to suggest that elm scolytids might act as vectors when he isolated *O.ulmi* from their breeding galleries, and shortly afterwards Wollenweber & Stapp (1928) observed *O.ulmi* synemata in pupal chambers. However, it was Betrem (1929) who realised the significance of the behaviour of elm bark beetles in connection with their role as vectors, and suggested that beetles carrying *O.ulmi* spores could infect healthy elms when feeding in twig crotches. This was demonstrated experimentally by caging *O.ulmi* contaminated scolytids on young elms (Fransen, 1931; Fransen & Buisman, 1935).

The intercontinental spread of Dutch elm disease continued when it was introduced from Europe to the United States in the late 1920s on imported elm veneer logs (Beattie, 1933), and to Canada probably in 1944 (Pomerleau, 1961). Due to the greater susceptibility of native American elms, especially the predominant white or American elm, *U.americana* L., (Smucker, 1941; Gibbs *et al.*, 1975) infection rates were were high, and there was no evidence of a decline as seen in Europe (Gibbs, 1978b). By 1961 the disease had spread through most of the natural range of elm in North America (Holmes, 1962) and also to the west of the Rockies in city plantings, first reported from the Pacific coast in 1973 (Partridge & Weir, 1974).

In the late 1960s serious outbreaks of Dutch elm disease were observed in southern England and shown to be caused by a new form of *O.ulmi* (Gibbs & Brasier, 1973). This new form was termed the 'aggressive' strain (*sensu* Vanderplank, 1968), because of its greater pathogenicity to European elms compared to the endemic 'non-aggressive' strain. Since the term strain was first used in this context it has become increasingly ambiguous, and so the different forms of *O.ulmi* will be referred to as subgroups. The significance of the differences at the subgroup level will be discussed later.

The presence of the highly pathogenic aggressive subgroup has been of great significance to the development of Dutch elm disease, since it has been responsible for the devastating epidemics of recent

decades. Initial examination of aggressive subgroup isolates from the epidemic areas in England revealed similarities in pathogenicity and cultural characteristics to isolates from North America (Gibbs & Brasier, 1973). Furthermore, the aggressive subgroup was isolated by Brasier & Gibbs (1973) from the xylem and bark of rock elm logs *(U.thomasii)* imported to Southampton docks from Canada, who also pointed out the proximity of the initial disease foci in Gloucestershire, south Essex and south Hampshire to the ports where rock elm had been entering Britain in the 1960s, namely Avonmouth, Liverpool, London and Southampton. Largely on the basis of this evidence it was concluded that the aggressive subgroup had been imported from North America (Brasier & Gibbs, 1973), and parallel investigations demonstrated that it was unlikely to have arisen from within the non-aggressive subgroup population already present in England (Brasier & Gibbs, 1976).

Further investigation of *O.ulmi* isolates from many parts of Europe, southwest Asia and North America revealed that the aggressive subgroup exists as two distinct races (Brasier, 1979), the North American race (NAN) and the Eurasian race (EAN). The NAN race was the aggressive subgroup imported from North America which subsequently spread eastwards across Europe, whereas the EAN race was found to be causing a second wave of disease spreading westwards from a probable origin in eastern Europe (Brasier, 1979, 1983a). Although there are clear differences between the NAN and EAN races there is sufficient similarity for them to be considered as races of the same species, in the broad sense of separately evolved populations with significant genetic differences. However, the non-aggressive subgroup is widely different from both the NAN and EAN aggressive races, and the aggressive and non-aggressive can be regarded as subspecies (Brasier, 1982).

The characteristics of the subgroups have been described in detail by Brasier (1982, 1986a, 1986b), and are summarised in Table 1.1. NAN and non-aggressive isolates are illustrated in Plate 1.2. Differences between the aggressive and non-aggressive subgroups have been found for all major characters examined, but the most important feature is the much greater pathogenicity of the NAN and EAN aggressive. The aggressive subgroup can kill fairly large trees of European elm species (eg *U.glabra*, *U.procera* and *U.carpinifolia*) in a single season, and will recur in the following season if the tree does

Table 1.1 Characteristics of the Three Subgroups of O.ulmi

Character	Non-aggressive subgroup	NAN aggressive	EAN aggressive
Pathogenicity (% defoliation);			
On clonal Ulmus procera	10–35%	80–100%	60–100%
On U x Commelin	0%	65–90%	45–85%
Cerato-ulmin production index	0–8	457–2668	320–1830
Cell wall degrading capacity	Poor	Good	Good
Optimum temperature for growth	c.30°C	20–22°C	20–22°C
Radial growth rate (mm/day at 20°C)	c.2.0–3.1	c.3.2–4.8	c.3.1–4.4
Colony morphology on MEA	Smooth waxy, or relatively undifferentiated aerial mycelium	Fibrous striate petaloid	Less striate and petaloid, often lobed and uneven
'Up-mut' colony dimorphism	Present	Absent	Present
Fertility barriers with other subgroups	Accepts NAN and EAN	Rejects non-aggressive Accepts EAN	Rejects non-aggressive Partially rejects NAN

Brasier, 1986a, using unpublished data of Richards, Takai & Brasier, and data from Brasier, 1977, 1979, 1982, 1984; Brasier, Lea & Rawlings, 1981; Scheffer & Elgersma, 1982; Svaldi & Elgersma, 1982; Takai, 1974, 1980. Differences in soluble protein and isozyme patterns have been demonstrated by Jeng & Hubbes (1983) and Bernier et al. (1983).

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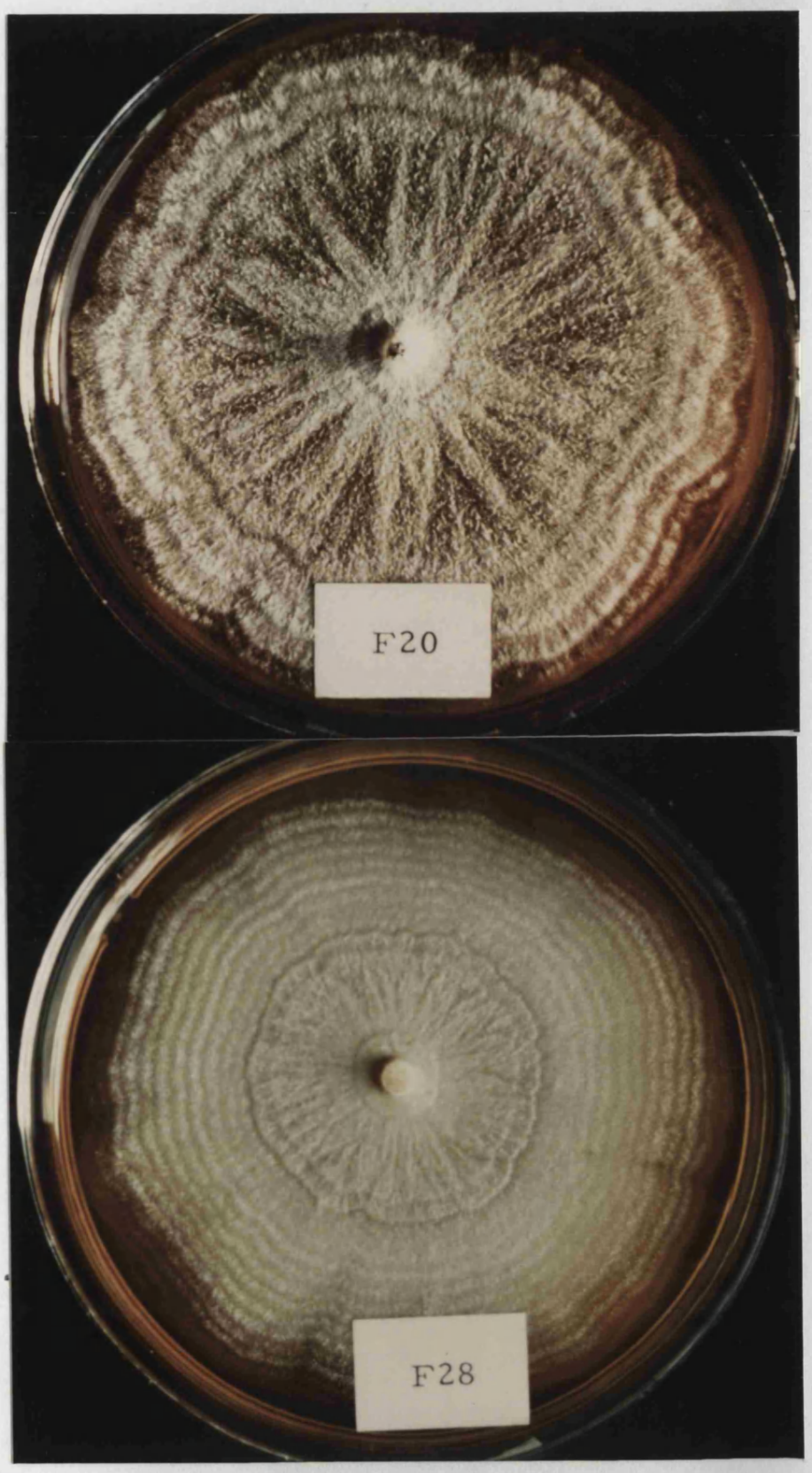
Plate 1.2 Isolates of the NAN (top) and Non-Aggressive Subgroups (below) grown on MEA (Courtesy of J.F. Webber)



not die. The non-aggressive subgroup usually causes only limited branch dieback and does not recur, although it is able to kill highly susceptible American elm, and multiple infections resulting from very heavy beetle feeding may kill more resistant species.

In culture the two major subgroups can be readily distinguished by their morphology and temperature growth relations. On 2% malt extract agar (MEA) the non-aggressive subgroup shows a range of morphology from waxy and appressed, to a moderately dense, only slightly differentiated aerial mycelium. The aggressive subgroup has a denser aerial mycelium, with a fibrous striate-petaloid appearance and develops a much more distinct diurnal zonation on exposure to daylight (Plate 1.2). The aggressive is faster growing up to about 27.5 ℃ , with an optimum of 20-22℃ and a maximum of 32-33℃ , whereas the non-aggressive has an optimum of 30℃ and a maximum of 35℃ (Brasier *et al.*, 1981).

The NAN and EAN aggressive races are not so easily distinguished in culture, although the EAN tends to have a more uneven and less differentiated aerial mycelium and a slightly slower growth rate. The EAN also shows a wider range and lower mean for pathogenicity (Brasier, 1986b).

A characteristic feature of EAN isolates is a mycelial dimorphism, named the 'Up-mut' factor by Brasier (1982). Colonies on agar media may show either wild-type striate-petaloid morphology or the slower growing uniform powdery, slightly appressed and undifferentiated morphology of the 'Up-mut'. Both forms will sector out quite unpredictably from the other, and subculturing from the wild-type to a fresh agar plate can give rise to either wild-type or 'Up-mut' mycelium, and *vice versa*. Control is almost certainly nuclear and the transition may be brought about by subtle nutritional differences in the medium (Brasier, 1986b). The 'Up-mut' factor does not occur in the NAN, although it is also a characteristic feature of the non-aggressive subgroup.

There is a complex of fertility barriers between the three subgroups. Both the NAN and EAN aggressive reject the non-aggressive almost totally, although the non-aggressive will accept either (Brasier, 1977, 1978). The NAN accepts the EAN, but the EAN will only partially accept the NAN (Brasier, 1979), providing a reliable method for separating them. The progeny of aggressive x non-aggressive crosses show a very wide range of morphologies which

cannot be classified into parental types, and show a wide range of growth rates and pathogenicities with the means usually lying below that of the parents (Brasier & Gibbs, 1976; Brasier, 1977). This is consistent with considerable genetic divergence between the aggressive and non-aggressive subgroups, with hybridization resulting in disruption of polygenic systems controlling major characters such as growth rate and pathogenicity.

Further evidence indicating complete ecological separation of the aggressive and non-aggressive subgroups comes from two sources. Firstly, from experiments using mites as fertilising agents (Brasier, 1978), where the mites efficiently fertilised NAN protoperithecia with NAN spores, but failed to fertilise NAN protoperithecia with non-aggressive spores and consequently no hybrid perithecia were produced. Secondly, despite the examination of thousands of wild isolates of *O. ulmi* from areas where the aggressive and non-aggressive subgroups were both present, no hybrid isolates have ever been positively identified (Brasier, 1986a), even though both subgroups have been isolated from the same piece of bark (Brasier & Gibbs, 1976; Lea, 1977). Evidence from the epidemiology of Dutch elm disease and the characteristics of the subgroups makes it possible to speculate about their evolutionary relationships and the origins of the various epidemic events of this century. The disease probably originates from eastern Asia (Heybroek, 1966) and was introduced to western Europe and later to North America, resulting in the epidemics of the 1920s and 1930s. It is thought that the earlier epidemics were caused by the non-aggressive subgroup, although it is not possible to be certain from contemporary descriptions, and the conditions leading to the development and subsequent decline of the epidemic in Europe are not understood. However, it seems unlikely that the aggressive subgroup was present at this time judging from its high pathogenicity and the continuation of high infection rates in the current epidemics. It also seems improbable that the two major subgroups could have occurred together before the current epidemics, since wherever the aggressive has been introduced, the non-aggressive has declined rapidly, probably to eventual extinction (Brasier, 1983a, 1986a; Houston, 1985).

The aggressive may well have arisen allopatrically from the non-aggressive or a common ancestor in eastern Europe (Brasier, 1986b), evolving into the EAN and spreading westwards across

Europe. The non-aggressive was presumably replaced at this centre of origin, but temporarily escaped extinction by an earlier introduction to western Europe and then North America. Brasier (1986b) has suggested that an EAN-like form could have been introduced to the American midwest from the eastern European origin in about the 1940s and evolved into the NAN, although McNabb (1974) has speculated that the NAN originated from within the non-aggressive population in the American midwest. However, it is necessary to hypothesise a fairly recent common origin for the NAN and EAN races because of the degree of similarity between them, and their present geographical distribution fits more easily with an origin in Europe.

Regardless of the origin of the NAN aggressive, changes in its distribution and the decline of the non-aggressive subgroup in North America (McNabb, 1974; Gibbs *et al.*, 1979; Houston, 1985) show that it has spread eastwards from the American midwest causing a renewed epidemic. The subsequent introduction of the NAN to Europe via Britain has now lead to the presence of the aggressive subgroup over most of the continent, although the westward spreading EAN epidemic would probably have achieved this alone (Brasier, 1983a).

In view of the history of intercontinental spread of Dutch elm disease by man and the apparently rapid evolutionary change in *O.ulmi* it is important that the putative eastern Asian origin should be found. More complete knowledge of variation in the pathogen is an essential background to breeding programmes for resistant elms, and for understanding and predicting future changes in the pathogen. Brasier (unpublished data) has examined a single Himalayan isolate of *O.ulmi* which differs from all three subgroups, and has demonstrated very strong fertility barriers, comparable to those between the aggressive and non-aggressive subgroups, between it and the NAN, EAN and non-aggressive. This isolate may represent an ancestral form of *O.ulmi*, perhaps more closely related to less ecologically specialised saprotrophic Ophiostoma species.

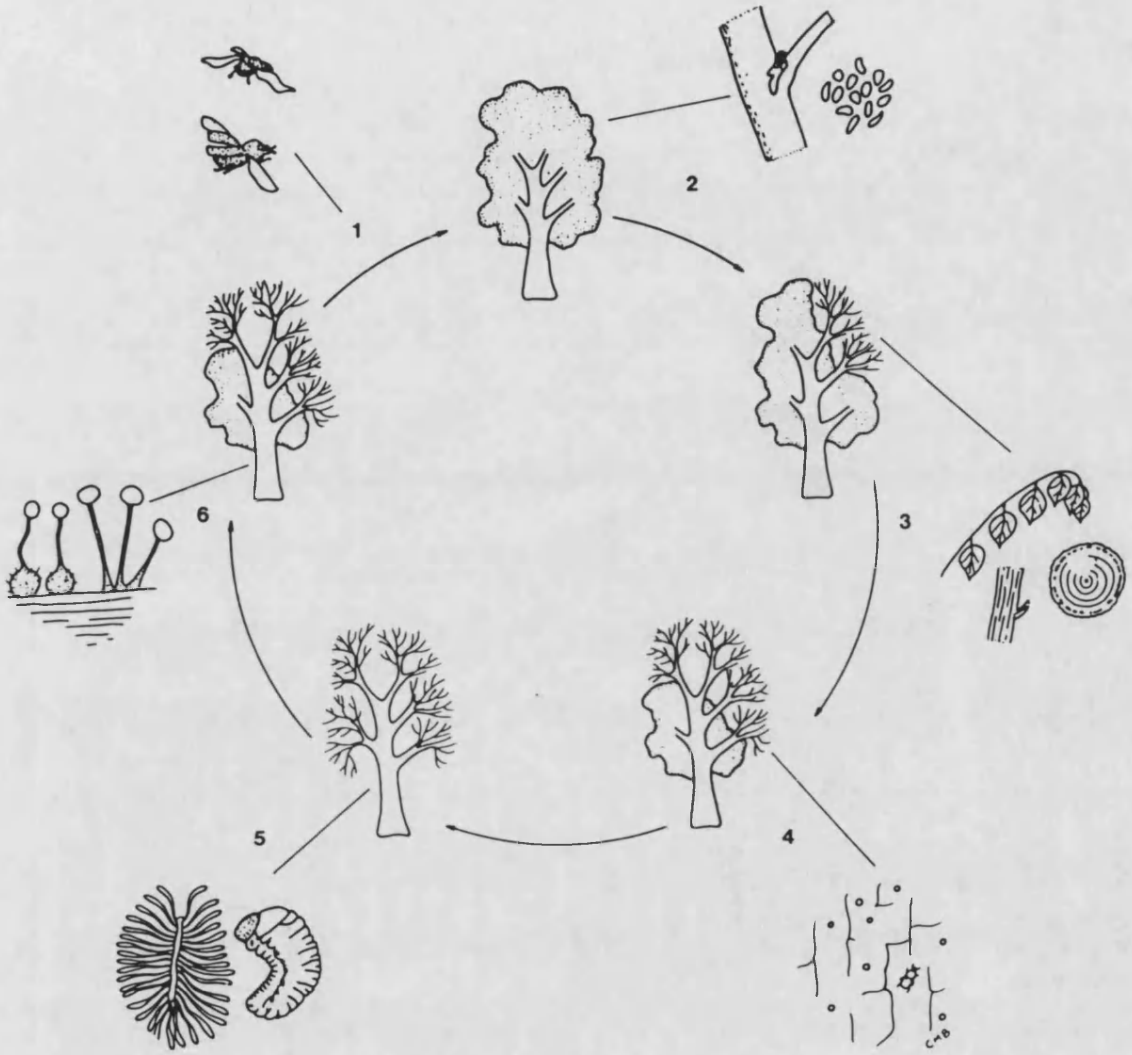
1.2 BIOLOGY OF DUTCH ELM DISEASE

The disease cycle is a complex one, with numerous interactions between the pathogen, vector and host. It is summarised in Figure 1.1. Trees may become infected when adult vector beetles emerge in late spring and early summer carrying *O.ulmi* spores, and fly to healthy elms to feed in the crotches of small twigs. Spores may be deposited in the feeding wound leading to the establishment of a short mycelial phase, and infection is initiated if the pathogen gains access to the vascular system via exposed xylem vessels. Once the pathogen is established it can spread rapidly through the tree causing external symptoms of wilting, defoliation (Plate 1.3) and dieback of branches, or death of the whole tree. Internally, infected vessels are stained dark brown or black, seen in cross-section as dark spots or a complete discoloured ring, and as streaks when the bark is removed. The beetles breed in the inner bark of dying or recently dead trees and branches (Plate 1.4), the females introducing *O.ulmi* as they excavate galleries and lay eggs. The bark becomes thoroughly occupied by larval galleries, *O.ulmi*, and other bark flora and fauna, in a long saprotrophic phase lasting from 2-10 months until the next generation of beetles emerges.

1.2.1 Biology of the Vector Beetles

In Europe the principal vector is *Scolytus scolytus* F., the larger elm bark beetle, although *S.multistriatus* Marsh., the smaller elm bark beetle, is also important. Other *Scolytus* species are involved in some areas, for example the native *Hylurgopinus rufipes* Eichh. in North America, although it is a less important vector than *S.multistriatus* which has been introduced from Europe (Chapman, 1910). The biology of *S.scolytus and S.multistriatus* has been investigated in some detail, principally by Fisher (1931, 1937), Fransen (1939), Collins (1941) and Beaver (1966, 1967). Most of the work concerned populations which had not been subjected to the selection pressures associated with epidemics caused by the aggressive subgroup. The major ecological difference between the two species are the slightly later emergence of *S.multistriatus*, usually 2-3 weeks after *S.scolytus*, and the size of material chosen for breeding. *S.scolytus* selects branches over 60 mm in diameter and *S.multistriatus* tends to be found in smaller branches, although there is considerable overlap. As the common names suggest *S.scolytus* is larger than *S.multistriatus*, with body lengths of 4-6 mm and 2.5-3.5 mm respectively.

Figure 1.1 Life Cycle of Fungus and Beetle in Dutch Elm Disease



1 Adult beetles emerge in early summer from the bark of dead and dying elm, carrying spores of O.ulmi

2 Beetles feed in the twig crotches of healthy elms

3 As a result of beetle feeding the pathogen may enter the xylem. Infected twigs wilt and show characteristic streaks or spots

4 Trees weakened by disease become breeding sites for beetles

5 Beetle larvae cut galleries in the bark

6 O.ulmi fruits in the breeding galleries

(From Webber & Brasier, 1984, after Peace, 1962)

Plate 1.3 Early Disease Symptoms on English Elm (Forestry Commission)

Plate 1.4 Early Stage Scolytus multistriatus galleries in the bark of a dying elm

It is important to note that both species can only breed in recently dead or dying elms, although they may attack severely drought stressed trees (Gibbs & Greig, 1977). In the absence of Dutch elm disease most breeding material would be provided by storm damage and disturbance by man, such as felling trees for timber and firewood.

When the adult beetles emerge they are sexually immature, but feeding in twig crotches is not essential for complete development of the gonads (Fisher, 1937; Beaver, 1967), despite its frequent description as maturation feeding. Beetles may fly directly to suitable breeding material and complete their sexual development while excavating galleries in the inner bark. Twig crotch feeding probably serves to prolong survival during adverse conditions and flight dispersal (Kirby & Fairhurst, 1983), providing a source of nutrients and water.

Beetles probably recognise elms by a combination of physical characteristics and host volatiles (Baker & Norris, 1968). Fransen (1939) observed that beetles tend to aggregate on particular trees, and it has been demonstrated that pheromones are produced to attract other beetles to feeding sites and breeding material (Peacock *et al.*, 1971; Borden & King, 1977).

Females of both *S. scolytus* and *S. multistriatus* make vertical galleries in the phloem of about 10-30 mm in length usually at the cambium and scoring the sapwood to a depth of about 0.5-2 mm, depositing eggs in niches at intervals along the galleries (Plate 1.4). Mating may take place in feeding grooves or at the entrance to the gallery. Males tend to be polygamous, moving from one entrance hole to another to mate with different females, and although *S. multistriatus* females probably make only a single gallery it is possible that *S. scolytus* may make more than one (Beaver, 1967; Kirby & Fairhurst, 1983). The larvae cut galleries radiating out from the maternal gallery, developing through five instars. Fecundity varies according to the suitability of the breeding material and climate (Kirby & Fairhurst, 1983), but even in pre-epidemic populations of *S. scolytus* it seems that predators and parasites had little regulatory effect (Beaver, 1966). The availability of host material is probably of much greater importance.

The overwintering fifth instar larvae are usually fully grown and pupate in late spring as temperatures rise. *S. multistriatus* pupates only in the phloem (Beaver, 1967), forming a chamber either entirely within

the inner or outer bark or across both, but some *S.scolytus* larvae form cells in the sapwood, penetrating up to 10mm (Fisher, 1937; Beaver, 1967). The proportion of these pupal cells varies according to climatic conditions and the host species, with temperature probably the most important factor (Kirby & Fairhurst, 1983). More cells tend to be cut in northern England, and also to a greater depth.

The pupal phase lasts from 10-15 days and adults remain in the pupal chamber for several days until sclerotisation is complete, before tunnelling to the bark surface and emerging when conditions are suitable. Both species will emerge at about 17℃ , and have a threshold for flight of about 20℃ (Fransen, 1939). Smaller larvae and larvae from pupal cells in the sapwood develop more slowly and consequently emerge later, spreading the emergence period through June, July and August. In southern England *S.scolytus* usually has a partial second generation which emerges from mid-August to mid-October. *S.multistriatus* only rarely has a second generation in England, although in warmer climates there may be a second and partial third generation (Collins *et al.*, 1936).

1.2.2 Disease Transmission

The number of beetles carrying *O.ulmi* spores and the size of the sporeload are major factors in determining the likelihood of infection. Webber & Brasier (1984), in a detailed investigation of the transmission of Dutch elm disease, have reported a large reduction in the percentage of beetles carrying spores after emergence and flight compared to beetles taken from pupal chambers. This reduction is probably due to the combined effects of ultraviolet radiation, desiccation and physical loss of spores. The sporeloads of single *S.scolytus* adults were found to vary from zero to greater than 20000, with most beetles carrying 250-2500 spores, whereas *S.multistriatus* carried significantly fewer spores, probably due to its smaller size. This may in part explain the greater importance of *S.scolytus* as a vector.

The percentage of feeding grooves contaminated with *O.ulmi* was found to be only slightly lower than the number of beetles carrying spores after flight. This suggests a high success rate for establishment, although a feeding groove may be used by more than one beetle, increasing the chances of contamination. However, only 5% of feeding grooves lead to infection of the xylem, indicating a threshold sporeload for successful infection (Webber & Brasier, 1984). Further work by Webber (1987) has shown that infection is unlikely if less than

1000 spores are inoculated into an artifical wound.

Although these factors combine to make transmission a relatively inefficient process, the enormous vector populations during an epidemic ensure rapid disease spread. The disease can also spread via xylem anastomoses between the roots of adjacent trees (Verral & Graham, 1935), which can be a particular problem in closely planted street trees of clonal origin, and where trees have arisen from a common rootstock such as in hedgerows.

1.2.3 Pathogenesis

The factors leading to succesful invasion of the xylem are poorly understood. Colonisation of the xylem probably follows mycelial growth in the feeding groove, rather than direct spore contamination of xylem vessels exposed by beetle feeding activities (Webber & Brasier, 1984). Evidence from the rate of movement of spore inoculations (Banfield, 1941) and scanning electron microscopy (Scheffer & Elgersma, 1982) strongly suggests that once *O.ulmi* has entered the xylem it exists in a budding yeast phase. Spread from vessel to vessel must be achieved by growth through pit membranes, as the pores are too small to allow living cells to pass. However, since a gap of only 0.1 μm is sufficient to admit air to vessels under tension, leading to cavitation and inactivation (Zimmerman, 1983), in order to spread with the transpiration stream *O.ulmi* must be able to penetrate the pit membrane and produce conidia without causing air seeding. It seems unlikely that the enzymes necessary to break down the pit membrane could be controlled to permit a hypha to penetrate without eventually leading to cavitation. Nevertheless, penetration and the production of conidia or budding may be sufficiently rapid to allow propagules to reach the far end of the vessel before it cavitates (Zimmerman, 1983). The very long springwood vessels of elms (Liming, 1934) would allow the pathogen to spread rapidly in this way. Indeed, elms are most susceptible to infection in early summer when they are relying on a relatively small number of long, wide diameter springwood vessels for water transport, before smaller summerwood vessels are laid down (Parker *et al.*, 1941). There is also a correlation between short, small diameter vessels and disease resistance (Elgersma, 1970; McNabb *et al.*, 1970). Although it is obvious that

O.ulmi causes a disruption of the tree's water relations, the actual mechanisms are unclear and it is particularly difficult to separate the primary causes of pathogenicity from secondary effects.

There is good evidence to suggest that disease results from the interaction of fungal metabolites and the tree, and that the presence of hyphae and tyloses are not the primary causes of wilting by physically blocking xylem vessels. The number of blocked vessels is probably insufficient to cause significant water stress (Wilson, 1965), and Elgersma (1973) has reported that tylose formation is faster in resistant elms, and is probably a host defence mechanism to restrict spread of the pathogen rather than a cause of water stress. Vessels with considerable colonisation would already be non-functional due to air seeding, although cavitation might cause more disruption to water flow than is realised if it is the result of very limited and undetectable fungal penetration (VanAlfen & MacHardy, 1978; Newbanks *et al.*, 1983).

Considerable emphasis has been placed on the role of toxins in pathogenesis. However, evidence for their role *in vivo* was limited until a low molecular weight protein, named 'cerato-ulmin', was isolated from shake cultures of *O.ulmi* by Takai and co-workers (Takai, 1974; Takai & Richards, 1978). Internal and external disease symptoms have been reproduced by inoculation of elms with 'cerato-ulmin' (Takai, 1974; Takai & Hiratsuka, 1984), and it has been found in extracts from diseased xylem (Takai *et al.*, 1983). In general, the aggressive subgroup has been shown to produce more 'cerato-ulmin' *in vitro* than the non-aggressive subgroup (Takai, 1974, 1980), and it can be argued that the non-aggressive subgroup does not produce any 'cerato-ulmin' at all (Richards, Takai & Brasier, unpublished data). There is also some suggestion that a high molecular weight glycopeptide fraction, first isolated by Salemink *et al.* (1965), may have a role in plugging pit membranes and causing cavitation of vessels (VanAlfen & Turner, 1975; Scheffer & Elgersma, 1981).

Cell wall degrading enzymes are likely to be important in the penetration of pit membranes and allowing *O.ulmi* to spread around the tree. There have been several reports of the production of cellulolytic and pectic enzymes by *O.ulmi* (eg Beckman, 1956; Svaldi & Elgersma, 1982), and greater levels of pectic enzyme activity have been detected in fluid from diseased elms than healthy elms (Woods & Holmes, 1974). However, wall degrading enzymes produced by the host may be involved in the formation of tyloses, and it is not easy to distinguish between host and pathogen enzyme activity. Svaldi & Elgersma (1982) have also shown that enzyme preparations from the aggressive subgroup release more cell wall degradation products *in vitro*

than those from the non-aggressive. Scheffer & Elgersma (1982) observed greater vessel wall breakdown in trees inoculated with the aggressive compared to those inoculated with the non-aggressive.

The most important criteria for pathogenicity once an isolate has entered the tree are probably high levels of toxin production and the ability to spread rapidly within the tree. However, various other factors determining the outcome of competitive interactions in the saprotrophic phase and in feeding grooves will also play a major role in the overall success of a particular genotype (Brasier, 1986a).

1.2.4 The Saprotrophic Phase

The breeding activities of the beetles introduce *O.ulmi* to dying elm bark, initiating the long saprotrophic phase. This provides an opportunity for a variety of interactions between the pathogen, host and vector, and other components of the bark flora and fauna. As far as *O.ulmi* is concerned it must ultimately contribute to the sporeload of the next beetle generation to continue the disease cycle. To do this it must sporulate in the pupal chambers, since larvae shed their outer skins and intestinal linings when they pupate, and lose any *O.ulmi* which they had been carrying (Fransen, 1939).

It is important to realise that the bark is still living when it is colonised and has some resistance to fungal attack. Therefore some aspects of pathogenic ability are likely to be important (Webber, 1979; Webber *et al.*, 1987), and it is misleading to use the term saprotrophic to describe the initial colonisation processes.

During the early stages growth of *O.ulmi* follows behind the larvae, and it seems that the early larval instars must feed on living, uncolonised phloem tissue for continued development (Webber, 1979, 1981). However, as the gallery systems develop the bark becomes thoroughly colonised by larvae and *O.ulmi*, probably with some secondary dispersal by mites and other bark fauna (Brasier, 1978; Lea & Brasier, 1983). In addition to the *O.ulmi* inoculum introduced by the beetles there is a significant but variable contribution from the pathogenic phase released from the xylem underlying the bark (Webber & Brasier, 1984). Far from being locked in the pathogenic phase, *O.ulmi* is able to colonise the bark, probably assisted by the breeding activities of beetles. Webber & Brasier (1984) showed that sexual recombination takes place between genotypes originating from the pathogenic phase and genotypes brought in by the breeding beetles. Thus, feedback from the

pathogenic phase will be important in maintaining the pathogenic fitness of the *O.ulmi* population.

A sequence in the production of the different fruiting structures during the overwintering saprotrophic phase has been described by Lea & Brasier (1983). Mycelial conidia are soon produced by the fungus growing in early beetle galleries, and synnemata with sticky spore masses are formed shortly afterwards (Plate 1.1). The rapid production of conidia in this way probably facilitates secondary dispersal by grazing mites. Perithecia are seen more frequently as temperatures begin to fall in late autumn, with an associated decline in the production of conidia. This lead Lea & Brasier (1983) to suggest that ascospores produced by the perithecia act as an overwintering stage, in addition to providing a source of genetic diversity. However, the same sequence of fruiting can be seen in the galleries of beetles completing an entire generation during the summer, and may therefore be ontogenetic rather than environmentally induced (Webber *et al.*,1987). All three spore stages, particularly mycelial conidia, are again produced in pupal chambers in late spring, probably the result of recolonisation and renewed activity as temperatures rise.

Lea (1977), and in Brasier (1984), using morphological and growth rate characteristics, has shown that the bark becomes occupied by a complex three dimensional mosaic of different morphological types, suggesting considerable genetic diversity in the *O.ulmi* population. The relative proportions of the morphological types present in the population initiating the saprotrophic phase were not the same as those carried out by the next beetle generation. This strongly suggests that the saprotrophic phase is dynamic, resulting from the various interactions thought to take place between the initial colonisation of dying bark and the emergence of the next generation of adult beetles.

The relatively small areas occupied by each morphological type identified by Lea and the juxtaposition of different mating types would provide ample opportunity for recombination and intraspecific competition. Protoperithecia produced by the aggressive subgroup A type, which shows morphological differentiation for increased sexual fecundity (Brasier & Gibbs,1975b; Brasier,1977,1984), may be fertilised with conidia carried by mites. Mites could also spread ascospores within the bark resulting in the establishment of new genotypes. Perithecia can easily be found in beetle galleries

(Walter, 1939; Brasier & Lea, 1983; Webber & Brasier, 1984), and it seems likely that changes in the bark population result from the establishment of new genotypes following sexual reproduction, and the outcome of competitive interactions between different genotypes.

More direct evidence for the dynamic nature of the saprotrophic phase comes from experiments by Webber & Brasier (1984). A tree was inoculated with a fungicide tolerant marked isolate and beetles naturally contaminated with *O.ulmi* allowed to breed in the bark. The frequency of recovery of marked isolates from the bark increased over the winter, and marked *O.ulmi* spores were found on beetles emerging in the spring. Most of the marked isolates recovered were of the same genotype as that inoculated into the tree, but some were shown to be products of recombination with genotypes brought in by the breeding beetles.

Contact between different mycelia during the saprotrophic phase will also provide the opportunity for spread of the cytoplasmic virus-like, ds-RNA associated disease of *O.ulmi*, the d-factor, (Brasier, 1983b). The d-factor has been shown to cause considerable reductions in growth rate, fertility and spore germination *in vitro*, and it seems likely that d-infected isolates will be at a disadvantage when competing in bark and also when causing infection from feeding grooves (Brasier, 1986a, c). However, d-infected isolates frequently lose their d-phenotype following inoculation and re-isolation from diseased xylem tissue, probably due to rapid conidiogenesis in the budding yeast phase. This suggests that isolates which have recently passed through the pathogenic phase may be free of d-infection, perhaps a fitness advantage when competing with isolates derived from the bark to bark cycle.

The d-factor can be readily transmitted *in vitro* via hyphal anastomsis, although transmission is greatly reduced by genetic differences which restrict viable hyphal anastomosis. There is some reduction in the expression of symptoms among single conidial isolates, but more significantly the d-factor is unable to pass through the sexual stage, and consequently single ascospore progeny are completely free of d-infection (Brasier, 1983b, 1986c). A close association has been demonstrated between the expression of symptoms following transfer of the d-factor from an infected to a healthy isolate, and the transmission of double stranded RNA (Rogers *et al*., 1986a, b.

1.3 DISEASE CONTROL

Measures to reduce the impact of Dutch elm disease have in practical terms been limited to various vector suppression methods and the treatment of particularly valuable trees. This is due mainly to the enormous epidemic momentum resulting from the very high pathogenicity of the aggressive subgroup towards European and North American elm species, the difficulties of dealing with large numbers of trees especially in rural areas, and the inaccessibility of the fungus in the xylem.

Control programmes based on scouting for diseased trees and then destruction of timber or bark to prevent scolytid breeding (sanitation felling) were carried out with reasonable success in the U.S.A. during the 1930s and early 1940s, but many were abandoned during the Second World War (Neely, 1973; Gibbs, 1978b). There are several more recent successful examples of similar programmes, mostly in urban areas or relatively isolated elm populations, such as Brighton and Hove (Greig & Gibbs, 1983), Guernsey (Riley, 1983), the Netherlands (Water, 1983) and several cities in the U.S.A. (eg Kostichka & Cannon, 1984; Baughman, 1985). In some cases the effectiveness of overall control programmes has been increased by additional measures, summarised by Burdekin & Gibbs (1974) and Sinclair (1978). These include insecticide spraying, curative injection with thiabendazole, pruning diseased branches, the prevention of root graft transmission, and in the last few years, the use of trap trees and pheromone traps (O'Callaghan & Fairhurst, 1983).

More recent investigations of treatments using biological agents (eg Scheffer, 1983; Mazzone & Peacock, 1985), have as yet shown only limited or doubtful benefits, and still suffer from the disadvantages of treating large numbers of individual trees. However, the severity of disease in northern and western Britain has undoubtedly been reduced through natural colonisation of elm bark by Phomopsis oblonga, making the bark unsuitable for beetle breeding and colonisation by *O. ulmi* (Webber, 1979, 1981).

Alternative control measures are required if remaining mature elms are to survive in the longer term, or if elms are to return as mature trees in the countryside where large numbers of suckers have grown from the roots of diseased trees (Greig, 1985). Replacement with resistant cultivars is an obvious answer for amenity trees in urban situations, and longstanding breeding programmes, especially in the

Netherlands and North America, have developed several moderately resistant cultivars incorporating resistance from Asiatic elm species (Burdekin & Rushforth, 1981). However, the replacement of susceptible elms in the countryside is clearly impractical, and perhaps the most hopeful prospect for future control is through some kind of manipulation of the *O. ulmi* population itself, either directly by genetic means or by altering the balance between *O. ulmi* and the d-factor (Brasier, 1983a. 1986a, c).

1.4 VEGETATIVE COMPATIBILITY AND SELF-NON-SELF RECOGNITION IN FUNGI

Vegetative compatibility will be used prominently in this work, and it is therefore necessary to introduce the concept and its postion in the study of fungal populations. Vegetative, somatic, or heterokaryon incompatibilty systems have been described in a wide variety of Basidiomycetes, Ascomycetes, Deuteromycetes and Myxomycetes (see reviews by Croft & Jinks, 1977; Rayner & Todd, 1979; Lane, 1981) and it seems likely that the system is universal in higher fungi (Rayner *et al.*, 1984). Incompatibility between any two isolates generally results from genetic differences at one or more incompatibility loci, and is therefore a heterogenic incompatibility system (Esser & Blaich, 1973). There are fundamental similarities with heterogenic incompatibility systems in plants and animals, which function primarily in self-non-self recognition (Esser & Blaich, 1973). Vegetative incompatibility should not be confused with sexual incompatibility, which is a homogenic system with incompatibility resulting when isolates are isogenic at one or more mating type loci.

Accumulating evidence has shown that populations of higher fungi are made up of vegetative or heterokaryon incompatibility (vc or hc) groups. There is no restriction to viable hyphal anastomosis between isolates of the same group, but isolates from different groups show various reaction phenomena and restriction of viable anastomosis when opposed in culture. It should be noted that in Basidiomycetes the reaction phenomena occur mostly between secondary dikaryotic mycelia which are themselves made up of genetically different but sexually compatible homokaryons. This means that in order to form the dikaryon potential vegetative incompatibility between the two component homokaryons must be overridden by sexual compatibility (Rayner *et al.*, 1984), and implies a similar override during sexual reproduction in Ascomycetes to allow plasmogamy and karyogamy in the ascogenous hypha. Although the results of vegetative incompatibility, such as zone lines in decaying wood and various reaction phenomena between opposed isolates in culture, have been frequently described since the 1930s, the implications for the structure of fungal populations were largely unrecognised until the broad concept of fungal individualism was developed by Rayner and Todd (Rayner & Todd, 1979; Todd & Rayner, 1980). Notable exceptions were the work of Verrall (1937), Adams & Roth (1967)

and Mylyk (1976), who clearly recognised that the reaction phenomena between isolates of *Fomes igniarius*, *F.cajanderi* and *Neurospora crassa* respectively, were the result of genetic differences between individual mycelia. In addition, several other workers have used reaction phenomena to recognise genetically distinct mycelia amongst field isolates (eg Mounce,1929; Campbell,1938; Childs,1963; Barrett & Uscuplic,1971).

However, the observation of hyphal anastomosis in higher fungi, and in particular the work of Buller (eg Buller,1931), lead to wide acceptance of the concepts of heterokaryosis and the unit mycelium. The fungal mycelium was seen as a co-operative unit containing genetically different nuclei in a common cytoplasm, resulting from unrestricted hyphal anastomosis, and giving fungal populations a unique organisation (Burnett & Partington,1957; Burnett,1976).

The unit mycelium concept and the discovery of the parasexual cycle (Pontecorvo,1956) provided an attractive explaination for the diversity and adaptability of many fungal species, especially plant pathogenic homothallic Ascomycetes and Deuteromycetes. However, it was recognised by several workers (eg Parmeter *et al.*,1963; Moore,1964) that there was very little evidence for these phenomena outside of the laboratory. In a review of the many reports of heterokaryosis, Caten & Jinks (1966) concluded that it was rare even in homothallic Ascomycetes and Deuteromycetes, and where it did occur it was restricted to within groups of genetically similar isolates by heterokaryon incompatibility systems. They suggested that heterokaryons successfully synthesised in the laboratory were mostly forced between complementary auxotrophic mutants growing on minimal media on which neither homokaryotic component would be able to grow alone. If unforced they were only formed between isolates of the same compatibility group.

Vegetative and heterokaryon incompatibility have been shown to be under polygenic and possibly multiallelic control, and estimates of the number of *het* or *vic* loci involved have been made for several species (Garnjobst,1953,1955; Jinks & Grindle,1963; Jinks *et al.*,1966; Mylyk,1976; Anagnostakis,1982; Croft & Dales,1984; Puhalla & Spieth,1983,1985).

The number of vegetative incompatibility groups found in a particular species seems to reflect the mating system and the balance between sexual and asexual reproduction and dispersal. In heterothallic

species vegetative incompatibility does not affect sexual compatibility or restrict genetic exchange, and there is generally considerable diversity for vegetative incompatibility type in natural populations. However, in homothallic or imperfect species vegetative incompatibility may well have the effect of restricting genetic exchange, and different vegetative incompatibility groups may therefore be evolving separately, as has been suggested for *Aspergillus nidulans* (Croft & Jinks, 1977). There is some evidence that the genomes of different heterokaryon incompatibility groups of *A. nidulans* are unbalanced when recombined (Croft & Jinks, 1977), which is consistent with genetic divergence. Where populations are composed of large geographically dispersed vegetative incompatibility groups there is some genetic variation within the groups, presumably resulting from accumulated mutations and possibly recombination through a parasexual cycle. The widespread occurrence of vegetative incompatibility systems has lead to the development of the individualistic mycelium concept (Rayner & Todd, 1979; Todd & Rayner, 1980; Rayner *et al.*, 1984). Vegetative incompatibility effectively defines an individual, such that mycelia of compatible isolates can anastomose to form a single unit, and the mycelia of incompatible isolates are able to maintain their integrity by preventing viable hyphal fusions. Caten (1972) demonstrated that vegetative incompatibility restricts the transfer of harmful cytoplasmic factors, and similar effects have since been reported in other fungi, particularly in *Endothia parasitica* (Anagnostakis, 1983) and also in *O. ulmi* (Brasier, 1983b).

As understanding of the vegetative incompatibility system has increased it has become available as a useful and sensitive tool for the routine analysis of fungal populations, allowing individuals to be recognised and mapped in a variety of situations. This should lead to greatly increased knowledge of the fundamental characteristics of fungal populations, particularly applicable to plant pathology. Simple assessment could be made of the potential genetic diversity and the importance of sexual reproduction in a pathogen, and recognition of genetically different isolates would allow changes in population structure resulting from changes in selection presures, such as those imposed by host resistance and the use of fungicides, to be monitored and predicted.

1.5 PHYSIOLOGY AND CONTROL OF VEGETATIVE COMPATIBILITY IN *O.ULMI*

The characteristics of *O.ulmi* vegetative compatibility reactions have been described in some detail by Brasier (1984), with similar phenomena of mycelial proliferation and sporulation to those reported in other fungi (see Rayner & Todd, 1979). Five different types of vc reaction were recognised, based on observations of NAN and EAN isolates paired on elm sapwood agar. The reactions were classified according to the appearance of the zone of mycelial proliferation (the 'barrage') where the two colonies met, and the production of synnemata:

i. 'Compatible' reaction. Colonies either merge, or there is a very slight mycelial thickening at the junction. No associated synnemata. Rarely found except between NAN or EAN 'supergroup' isolates (see Chapter 1.6), or control pairings of the same isolate.

ii. 'Line-gap' reaction. A narrow gap (c. 1-3 mm) of sparse mycelium, without associated synnemata. Rarely found between wild isolates.

iii. 'Line' reaction. Thin mycelial line only 1-3 mm wide, without associated synnemata. Rarely found between wild isolates.

iv. 'Narrow' reaction. Narrow (c.5 mm) denser barrage, with synnemata formed on either side, but to a lesser depth than in wide reactions. Found occasionally in random pairings of wild isolates.

v. 'Wide' reaction. Wide (c. 10-15 mm) diffuse white barrage, with synnemata formed up to several cm on either side. Found frequently in random pairings of wild isolates.

In wide and narrow reactions synnemata, and perithecia where appropriate, are formed progressively further away from the barrage as the reaction develops. Using nuclear markers it has been shown that the synnemata in one isolate are produced by the opposing isolate, and this has been termed the 'penetration effect' (Brasier, 1984). Penetration is unequally bilateral in most pairings of wild isolates, but it may be equally bilateral, or completely unilateral. A sample of isolates paired in all combinations can be ranked in order of penetrating ability, with some isolates consistently strong and others consistently weak penetrators. The penetration effect is likely to have a major role during the saprotrophic phase, when different genotypes occupy adjacent areas of bark.

Experiments using nuclear and cytoplasmic markers have shown that penetration is almost certainly the result of mycelial

introgression (Brasier, unpublished data), rather than nuclear migration. Even when the isolates are fully compatible and there is no restriction to viable anastomosis, nuclei of one isolate cannot be detected more than 2-3 mm into the other. Similarly, when testing for heterokaryosis in *Fusarium oxysporum* and *F.moniliforme* using nuclear markers but without forcing, complementation of the two markers was restricted to a few mm either side of the junction between the colonies, and was observed only between marked isolates of the same vc group (Puhalla,1984a,1985; Puhalla & Spieth,1983,1985).

Investigation of mycelial interactions in the Ascomycete *Daldinia concentrica*, however, has indicated that the dense white aerial mycelium formed between incompatible isolates is heterokaryotic (Sharland & Rayner,1986). Hyphal tip isolations from this mycelium occasionally gave rise to colonies unlike either of the original interacting isolates, but which sometimes sectored into the two original isolate types. Reactions were classified according to the production of white aerial mycelium, and the characteristics of different reaction types interpreted as evidence for various patterns of nuclear migration, leading to either; mutual rejection, the formation of a heterokaryotic sector, or replacement of one nuclear type by another. This was compared to similar phenomena in Basidiomycete mating reactions, where genetically different nuclei become associated in the same cytoplasm to form a stable dikaryon. Therefore, in *D.concentrica* genetically different nuclei may be allowed some degree of access to an acceptor mycelium, but in other Ascomycetes, including *O.ulmi*, rejection may take place immediately and completely prevent nuclear migration. The genetic control of vegetative compatibility in *O.ulmi* has been investigated through a backcross series, using as the first set of parents two NAN aggressive isolates giving a wide reaction, and vc testing the progeny against both parents (Brasier,1984). In the first generation the progeny fell into two classes: one giving a wide reaction against the first parent and a narrow reaction against the second, and the other giving a narrow reaction against the first parent and a wide reaction against the second. It was concluded that a narrow reaction resulted when the two opposed isolates were isogenic at one vc locus, termed the *w* locus.

Backcross lines were continued from both original parents, using in each case perithecia from a narrow reaction. As expected, no further wide reactions were seen, but some line and line-gap reactions

were found and used to continue the backcross lines for a third generation. Some compatible reactions against the original and backcross parents were found in this generation, some of which were of opposite mating type, confirming that the mating type locus has no involvement in vegetative incompatibility. Progeny of parents giving a compatible reaction also gave compatible reactions against both parents.

Thus, in four generations differences at all of the vc loci involved were eliminated by selecting at each stage progeny giving the weakest reaction category to continue the backcross series. This suggests that the original parents were different for at least four vc loci. However, the diversity of vc types among wild isolates (see Chapter 1.6) and comparison with other fungi (eg Mylyk, 1976; Anagnostakis, 1982; Croft & Dales, 1984) suggest that vc in natural populations of *O.ulmi* is controlled by more than four loci.

Evidence from pairings of wild NAN aggressive isolates has suggested that the *w* locus is multiallelic. Groups of isolates have been recognised such that isolates from the same group all give narrow reactions against each other (ie they all have the same *w* allele), but pairings between groups give wide reactions (Brasier, 1984, 1986a). However, this does not exclude the possibility of multiple biallelic *w* loci. Variations in the appearance of each reaction category with different isolate combinations also suggest that there is some interaction with the genetic background (Brasier, 1984, 1986a).

The expression of vc reactions apparently depends on an epistatic hierarchy between the controlling loci. For example, narrow reactions are only expressed when the opposed isolates have the same *w* allele, and line-gap reactions are only expressed when the isolates are isogenic at all other vc loci. The various vc reactions therefore result from different levels of genetic difference, such that wide and narrow reactions result from differences at many vc loci, line-gap reactions result from only one or two differences, line reactions when there is a difference at a single locus, and compatible reactions when isolates are isogenic at all vc loci. This is an obvious corollary to the wide diversity of vc types and the high frequency of wide and narrow reactions in pairings of wild isolates (Brasier, 1984, 1986a, and see Chapter 1.6). In *O.ulmi* vegetative incompatibility can be seen as fulfilling several major functions:

i. The recognition of non-self, and the maintenance of territorial integrity by preventing viable hyphal anastomosis and the

formation of heterokaryons.

ii. Territorial invasion by the penetration effect. Although there is no evidence that a penetrated isolate is replaced, the enhanced sporulation for an isolate with strong penetrating ability, perhaps due to lysis of the hyphae of the weaker isolate (Brasier, 1984), may provide a considerable advantage especially in the saprotrophic phase.

iii. Promotion of outbreeding. Brasier (1984) has shown that more perithecia are produced in wide reactions, where penetration is greatest, and where genetic differences between the two isolates will also tend to be greater.

iv. Prevention of the spread of the d-factor (Brasier, 1983b, 1984, 1986c). Transmission is virtually unrestricted in compatible reactions, slightly restricted in line-gap and line reactions, greatly restricted in narrow reactions, and almost completely restricted in wide reactions.

1.6 THE POPULATION BIOLOGY OF *O.ULMI*

The existence of the aggressive and non-aggressive subgroups and the further division of the aggressive subgroup into the NAN and EAN races has already been described. A conspicuous feature of the population structure at the subgroup level during the recent Dutch elm disease epidemics has been the decline of the non-aggressive when forced to compete with the aggressive. The decline of the non-aggressive from the original outbreak areas in England has been summarised by Brasier (1983a), together with data from the Netherlands and Eastern Europe. A similar pattern has been recorded from many other areas (Gibbs *et al.*, 1979; Brasier, 1986a; Houston, 1985). The most complete data come from the Netherlands (Brasier, 1983a), where the non-aggressive subgroup declined rapidly at a rate of about 10% each year from 1974 to 1980. In England the decline over the same period has been less rapid, but the non-aggressive subgroup was already absent from samples in 1978, suggesting an earlier origin for the epidemic in England. The non-aggressive has not been recorded in England since 1981 (Greig, 1982), even though several hundred *O.ulmi* isolates have been examined since this time. It can be concluded that the non-aggressive subgroup will be replaced by the aggressive wherever they occur together (Brasier, 1983a, 1986a). The level of genetic diversity and the recognition of different genotypes within the subgroups has been investigated using the fungus' vegetative incompatibility system (Brasier, 1984, 1986a). Populations have also been analysed for the relative frequencies of the two mating types.

Using vegetative incompatibility to analyse 'worldwide' and local pathogenic phase samples of the non-aggressive subgroup Brasier (1984) found a considerable diversity, with almost every isolate of a different vc type. The A and B mating types were found in equal frequencies. In contrast, when similar samples of the NAN and EAN aggressive were analysed particular vc groups in each (the NAN and EAN 'supergroups') were found to make up a considerable proportion of the samples. The two supergroups were distinct. Further investigation of some of the remaining isolates from each sample showed that almost all of them were of a different vc type, and suggested a high level of genetic diversity in the heterogeneous or non-supergroup component of each sample. All isolates of both supergroups were B mating types, but A mating type isolates were

found in the remainder of the sample at an overall frequency of much less than 50%.

The proportion of supergroup isolates in a particular sample varied according to epidemic status (Brasier, 1984). At advancing epidemic fronts the supergroup could comprise over half of the total sample, with a correspondingly low A mating type frequency. However, in post-epidemic areas the supergroup frequency was much lower and the A mating type frequency higher, although still less than 50%. More detailed investgation of NAN aggressive populations at fresh epidemic fronts in Spain and Portugal has shown that the population structure can be near clonal, with single vc groups predominant (Brasier, 1988). However, a rapid change to a more heterogeneous population structure was found immediately behind the epidemic fronts, presumably as a response to changes in selection pressure.

The aggressive subgroup populations are therefore thought to be made up of an asexually reproducing component, the supergroup, and a sexually reproducing heterogeneous component. There is no reason to suggest that the supergroups are reproductively isolated from the rest of their respective populations. They are presently assumed to arise in response to the selection pressures acting during rapidly developing epidemics on susceptible host populations (Brasier, 1984, 1986a), but the availability of host material also has some effect on the degree of this pressure, shown by variation in supergroup frequency according to epidemic status.

The unequal mating type ratio in populations of the NAN and EAN aggressive can be explained if the slower growth rate and lower pathogenicity of A mating type isolates are assumed to result in a selective disadvantage (Brasier & Gibbs, 1975b; Brasier, 1977, 1984). However, this must be balanced by the advantages of sexual reproduction and the morphological and physiological differentiation shown by A mating type isolates for increased sexual reproduction. The extent of variation for vc type strongly suggests that populations of all three subgroups are regularly outcrossing. In comparison to the NAN and EAN aggressive, the non-aggressive subgroup population examined by Brasier (1984) showed neither subdivision into an asexually reproducing component nor sexual dimorphism.

1.7 OBJECTIVES OF THE PRESENT RESEARCH

The ultimate objective for much Dutch elm disease research would be to eventually restore the balance between host and pathogen, as existed with the non-aggressive subgroup following the epidemics earlier this century and before the introduction of the aggressive. However, a simple solution is unlikely in view of the complexity of the disease cycle, with its numerous interactions between host, pathogen and vector, and the actions of man introducing *O.ulmi* to susceptible elm populations. Hopefully, continuing research into the fundamental biology of Dutch elm disease may provide the means to restore the balance, and allow mature elms to once more become a valued and functional part of our environment.

The increasing knowledge of *O.ulmi* population dynamics can also provide a useful model for understanding the behaviour of other plant/necrotroph systems, and to develop principles for the responses of pathogens to changes in selection pressures. *O.ulmi* has several features relevant to this kind of research, such as the dramatic changes in the population structure at the subgroup level, and the subdivision of populations of the aggressive subgroup into asexually and predominantly sexually maintained components.

The destruction of elms by *O.ulmi*, although one of the more spectacular and widespread epidemics to have been initiated by man, is certainly not the first, and has important implications for other diseases. Further examples of introduced tree pathogens are chestnut blight caused by *Endothia parasitica*, which has several parallels to Dutch elm disease (Gibbs & Wainhouse, 1986), and oak wilt caused by *Ceratocystis fagacearum*. Oak wilt is presently limited to eastern North America and although serious has not been as destructive as Dutch elm disease, partly because it lacks a comparable vector system. However, its epidemic potential is probably as great should it link up with a suitable vector (Gibbs *et al.*, 1984). This could be brought about either by its introduction to Europe, or alternatively by the introduction to the U.S.A. of the European oak bark beetle *(Scolytus intricatus)*, which has a very similar biology to the elm bark beetles (Yates, 1984). More detailed knowledge of Dutch elm disease is of obvious relevance to this and other potentially serious exotic diseases. Investigation of vegetative incompatibility in *O.ulmi* has made it available as a powerful tool for more detailed studies of the population biology of the fungus. It is apparent that many of the interactions

likely to be important in determining the population structure at both the inter and intra subgroup level take place during the saprotrophic phase. However, the structure of saprotrophic phase populations has not been investigated using vegetative incompatibility, although Lea (1977) has shown that fully colonised bark is occupied by a mosaic of different morphological types.

The middle and later stages of bark colonisation, particularly by *O.ulmi* but also including other fungi and the vector beetles, have been investigated by Lea (1977) and Webber (1979). Little is known, however, about the the early stages of colonisation, when *O.ulmi* is establishing in dying bark in association with breeding beetles. This early stage will be critical in determining the eventual composition of the mosaic in fully colonised bark. Several factors are likely to be important, such as competition between genetically different isolates introduced to the bark by the beetles, competition with isolates feeding back from the pathogenic phase in the underlying xylem, and residual resistance of the dying bark to fungal invasion.

Investigations of the population biology of *O.ulmi* have concentrated on the aggressive subgroup, although the dramatic decline of the non-aggressive when competing with the aggressive has been examined in terms of the proportion of each subgroup in the overall *O.ulmi* population. More detailed information of the population structure of the non-aggressive subgroup, particularly in during the saprotrophic phase, is prerequisite to an investigation of the interaction between the two subgroups. A similar approach to that used for the aggressive subgroup could be used to examine the pattern of colonisation in bark, and the genetic diversity in both pathogenic and saprotrophic phase populations.

Investigation of the interaction of the aggressive and non-aggressive subgroups, and the factors leading to the replacement of the latter, should provide valuable information regarding those particular characteristics of the aggressive subgroup which have most contributed to its success. This information could then be used to predict the attributes required in a more moderately pathogenic form of *O.ulmi* able to compete with the aggressive subgroup. However, it is also possible that a less pathogenic form of the aggressive subgroup might develop naturally in response to post-epidemic selection pressures (Brasier, 1983a, 1986a). In areas where the NAN and EAN aggressive occur together hybrids have been found to make up a

considerable proportion of the *O.ulmi* population (Brasier, 1986b, d), and would thus provide a more diverse gene pool on which post-epidemic selection pressures could act. Alternatively, the pathogenicity of the aggressive subgroup might remain largely unchanged if a strategy of local disease flare-ups in an elm population reduced to small sucker regrowth proves to have long term stability (Brasier, 1983a).

Although there is a considerable amount of information regarding differences between the aggressive and non-aggressive subgroups, such as pathogenicity and temperature growth relations, the factors involved in the replacement of the non-aggressive subgroup have not been investigated. It is very likely that interactions taking place during the saprotrophic phase result in a reduced contribution by the non-aggressive subgroup to the sporeloads of the next generation of beetles, compared to the sporeloads of their parents. Many factors will be involved in the greater success of the aggressive subgroup, but the critical stages are probably; establishment in beetle galleries, capture and maintenance of bark 'territory', colonisation of pupal chambers, and sporulation in pupal chambers to contaminate the emerging beetles with the spores necessary to continue the disease cycle.

In the light of the above, the main objectives of the work presented here are:

1. To verify the population structure of the NAN aggressive during the saprotrophic phase using the pathogen's vegetative incompatibility system, and to compare the structure of pathogenic and saprotrophic phase populations on the same basis.

2. To investigate the early stages of colonisation of elm bark, and the factors leading to the establishment of a mosaic of genotypes.

3. To examine the physiology and control of vegetative incompatibility in the non-aggressive subgroup.

4. To investigate the structure of saprotrophic and pathogenic phase populations of the non-aggressive subgroup using the pathogens vegetative incompatibility system.

5. To investigate the ecological interaction between the aggressive and non-aggressive subgroups, and in particular the causes of the rapid replacement of the non-aggressive under field conditions.

6. Hopefully, to provide information which could be of use in restoring the balance between host and pathogen through direct or indirect manipulation of the *O.ulmi* population.

2 MATERIALS AND METHODS

2.1 SAMPLING AND ISOLATION METHODS

2.1.1 Pathogenic Phase Samples

Short lengths of twig were taken from trees infected as the result of beetle feeding in the current season, recognised by limited disease spread and xylem streaking in the current annual ring only. Disease resulting from root connections with adjacent trees, or from an infection initiated in the previous season was not sampled.

Isolations were made from twigs by cutting away the bark and plating out shavings of freshly exposed discloured xylem onto 2% MEA supplemented with cycloheximide and streptomycin (MEA+C+S, Appendix 1). Any *O.ulmi* growing out from the shavings after 4-7 days incubation at room temperature (c. 20℃) was subcultured onto MEA+C+S.

2.1.2 Saprotrophic Phase Samples

Slabs of bark at least 200 x 200 mm with recent beetle breeding at the required stage were carefully removed using a mallet and chisel. Isolations made as soon as possible, usually within a few days. Bark slabs were more easily removed from larger trees, but the availablity of suitable trees depended on local disease conditions. On Mersea Island, Essex, the source of most of the NAN aggressive samples, larger trees tended to die branch by branch over more than one year, and so suitable bark was usually patchily distributed and often awkwardly positioned. In Spain, the source of the non-aggressive subgroup samples, suitable bark was easily found in areas with high disease levels and large numbers of rapidly dying trees. In endemic areas, good sampling material was obtained from felled trees or those killed by disturbance rather Dutch elm disease.

The difficulties of bark sampling on Mersea Island could be reduced by putting out trap logs to attract beetles, positioned clear of the ground near trees with signs of recent emergence or excavation of maternal galleries. Once it had been established from the presence of entrance holes and frass that beetles were breeding in the bark the logs could be recovered from the field and isolations made at convenient times from freshly removed bark. It was also possible on occasion to fell branches of diseased trees with natural breeding and bring logs back to the laboratory. These logs also provided a source of beetles for other experiments.

Isolations were made from bark in three ways:

i. Chip isolations. The inner (cambial) surface of the bark was removed and a small chip of bark, c. 1-2 sq mm, plated out on MEA+C+S.

ii. Dilution series isolations. A 5 mm diameter core of inner bark was taken after freshly exposing the surface and ground up in a known volume of sterile distilled water. A dilution series made from the macerate and an appropriate volume of each dilution spread onto MEA+C+S.

iii. Synnematal stabs. Synnemata in maternal or larval galleries were brushed with a tungsten needle, and the needle stabbed onto MEA+C+S.

Any *O.ulmi* developing after 4-7 days incubation at room temperature in darkness was subcultured onto MEA+C+S.

Isolations from discrete gallery systems in otherwise living bark, such as those in Plate 1.4, could be carried out fairly simply after mapping each system and marking the positions of numbered isolations. However, a more complex procedure was necessary at later stages when the larval galleries of adjacent systems had met. Firstly, a traced map was made of maternal galleries and if possible of the outlines of associated larval galleries. Following the method of Lea (1977), a grid was then set out over the bark using map pins and cotton (Plate 2.1), and a chip taken from each intersection. A grid interval of 10 mm was preferred, but occasionally a 20 mm interval was used to cover a larger area of bark for a given number of isolations.

2.1.3 Isolations from Beetles

Isolations were made from dilution series of individually crushed beetles, modified from a method used by Webber (personal communication). Emerging beetles were collected singly in sterile universal bottles from logs kept in net bags of a mesh size sufficiently small to prevent beetles escaping. Each beetle was macerated in 5 ml of sterile distilled water, and 0.5 ml of the macerate spread onto each of four plates of MEA+C+S. A 1 ml aliquot of the macerate was transferred to 9 ml of sterile distilled water and 0.5 ml spread onto each of three plates of MEA+C+S. The series was continued to give dilution factors of $1x10^1$, $1x10^2$, $1x10^3$, and $1x10^4$. The number of replicates was sometimes reduced to three for the first dilution and two for successive dilutions, depending on the acceptable balance of speed and accuracy.

Since isolations were usually made to determine the number of

Plate 2.1 A 10mm Interval Grid Laid out over Fully Colonised Bark

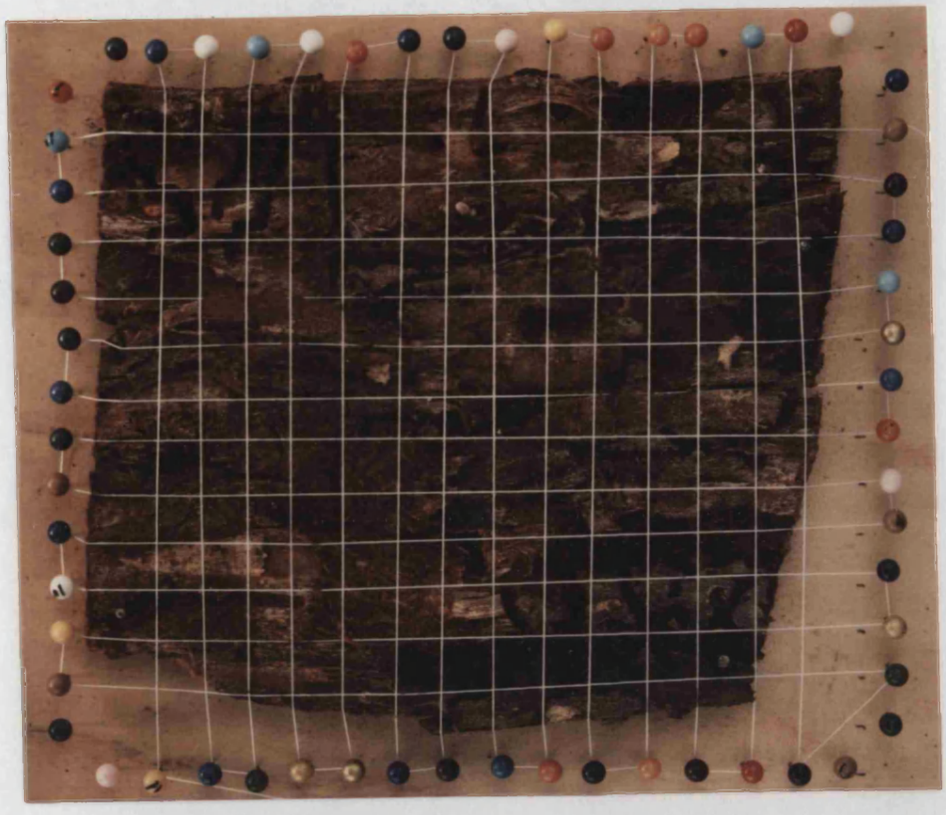
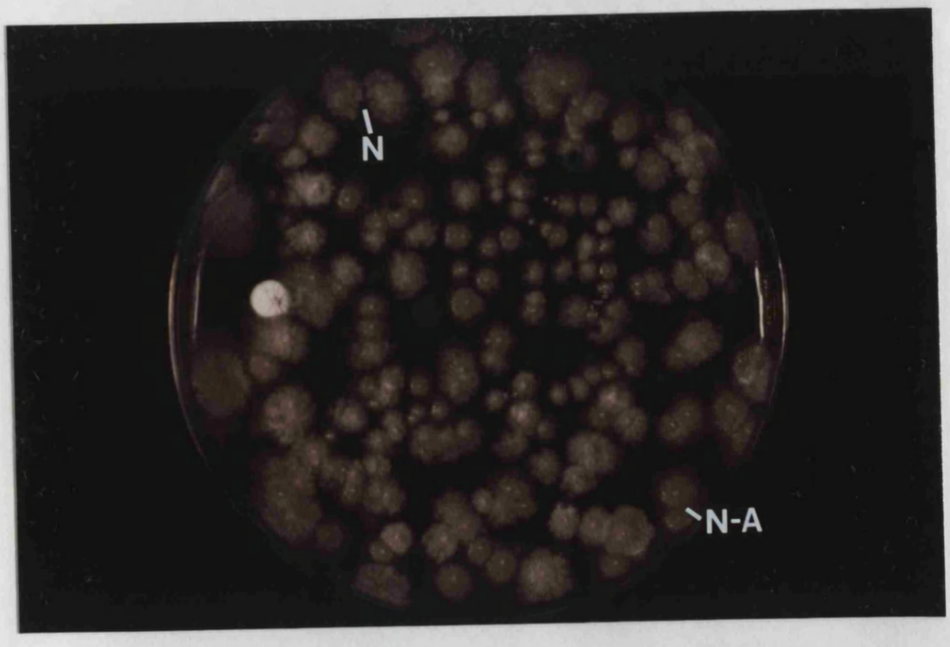


Plate 2.2 Dilution Series Isolation Plate from a Beetle Carrying both NAN (N) and Non-Aggressive (N-A) Spores



spores of the NAN and non-aggressive subgroups on each beetle, the dilution plates were incubated at 25 or 27 C in darkness to even out the growth rate differences between isolates of the two subgroups (Brasier *et al.*, 1981). Colonies were identified to subgroup and counted after 4-6 days. Identification was checked after further incubation at room temperature in diffuse natural light to allow colony differentiation. A dilution series isolation plate from a beetle carrying both NAN and non-aggressive spores is shown in Plate 2.2. When *O.ulmi* colonies were greatly outnumbered by those of other fungi or one subgroup greatly outnumbered the other, colonies which could not be positively identified were subcultured onto MEA+C+S and identification confirmed after several days incubation at 20°C.

2.2 GROWTH RATE MEASUREMENTS

Standard techniques for the measurement of radial growth rate and subsequent assessment of colony morphology (Brasier & Gibbs, 1973; Brasier, 1981) were used whenever it was necessary to characterise isolates in detail. A small inoculum taken from the edge of an actively growing colony was placed centrally on 2% MEA (Appendix 1), dispensed at 20 ml per plate. Two replicates were inoculated for each isolate and placed on different shelves to account for variation within the incubator. After 2 days incubation at 20℃ in darkness two diameters were measured for each colony along lines marked across the bottom of the plate, passing through the centre of the colony and approximately perpendicular to each other. A second measurement of the marked diameters was made after a further 5 days incubation, and a mean growth rate for each replicate calculated as radial increase in mm/day. Variations in incubation temperature and time, and the number of replicates were sometimes made according to the requirements of individual experiments.

Colony morphology was examined after another 10-20 days at about 20 ℃ in diffuse natural light. This allowed the characteristic features of diurnal zonation and patterns of aerial mycelium of each of the subgroups to develop (Plate 1.2).

Although the above method provided a positive means of identifying isolates of the NAN aggressive and non-aggressive subgroups, it was found that with practice they could be discriminated when first isolated from xylem and bark tissue or dilution plates. In some cases, such as when isolating from beetles, it was essential that identification could be made quickly and accurately or the number of isolations practicable for any experiment would be reduced.

2.3 VEGETATIVE COMPATIBILITY TESTS

2.3.1 NAN Aggressive

Isolates were opposed on elm sapwood agar (ESA, Appendix 1) with the inocula about 10 mm apart, and incubated at 20°C until the agar surface was covered, usually c. 7 days. Incubation was continued at room temperature in diffuse natural light to encourage the formation of synnemata and perithecia, and assessments for reaction patterns made after a total of 3-5 weeks (Brasier, 1981, 1984).

The above method was modified to allow a greater number of tests to be made more efficiently and rapidly. A total of 24 tests could be made by placing 16 inocula in a 4x4 pattern at c. 20 mm intervals on a single plate. This gave acceptably clear reactions, and although the clarity varied with different batches of agar it was always possible to at least tell compatible from incompatible wide or narrow reactions (Plate 2.3). Assessments were made after 5 days incubation at 20 °C in darkness, followed by 4-9 days at room temperature in diffuse natural light. Excessive development of synnemata tended to obscure the reactions, making the timing of assessments more critical than with a single test per plate.

The 4x4 pattern of 16 inocula per plate greatly simplified the analysis of isolations from grids laid over fully colonised bark. Tests were made of overlapping groups of 16 isolates, such that the edge row or column was repeated in adjacent groups of 16 isolates. In this way a picture could be built up of the pattern of different genotypes occupying the bark, although further series of tests were always necessary to establish relationships between separated genotypes.

2.3.2 Non-Aggressive Subgroup

Tests for vegetative incompatibility between isolates of the non-aggressive subgroup were made in essentially the same way as for the NAN. The clarity of reactions varied greatly due to the smaller numbers of synnemata and perithecia produced by the non-aggressive subgroup, and to a greater sensitivity to variation in the elm sapwood medium. Attempts were made to improve the medium, particularly by adding MEA and linoleic acid, and by milling the elm twigs with bark intact (Appendix 1). Including bark and MEA presumably increased the levels of nutrients. *O.ulmi* requires fatty acids for production of synnemata and perithecia (Hubbes, 1975; Marshall *et al.*, 1982, and linoleic acid is probably the most stimulatory when added to artificial media. Concentrations greater

Plate 2.3 VC Test of NAN Isolates in a 4x4 Pattern on ESA

Showing compatible (c), narrow (n) and wide (w) vc reactions

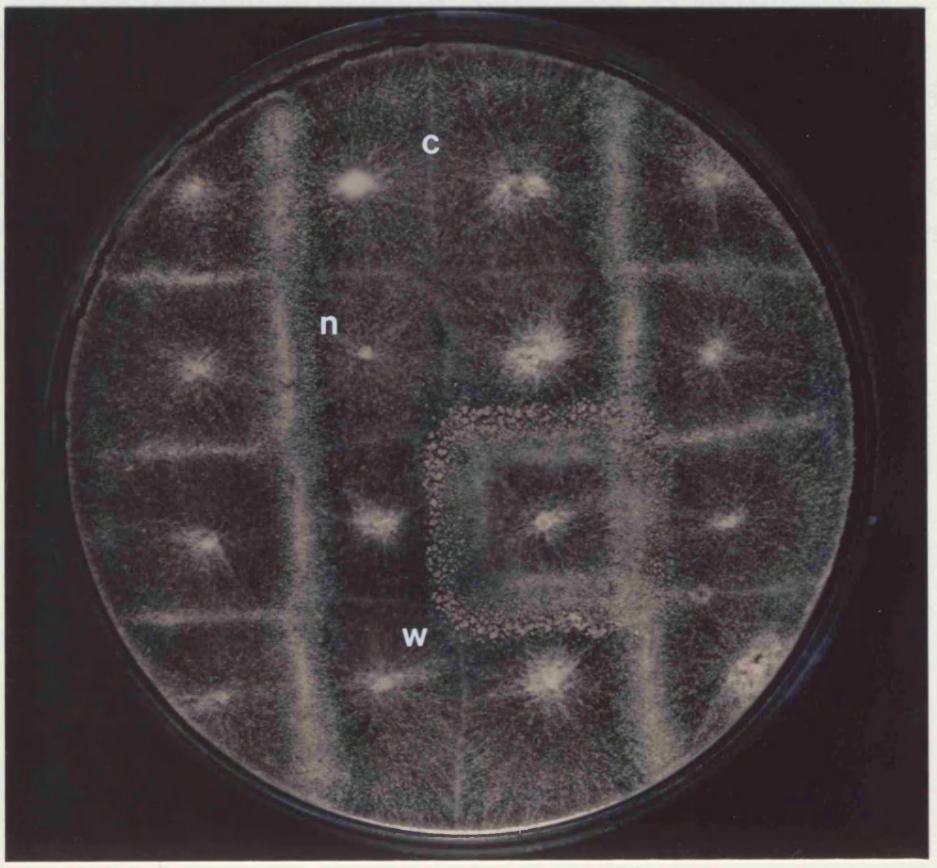


Plate 2.4 Lesions Formed by Inoculation of Elm Bark with NAN Isolates

than 2 ml/l were found to be inhibitory. The amendments usually improved the reactions, particularly by increasing the production of synnemata, but there was still considerable variation between batches of agar. This variation was assumed to be the result of differences in the elm sapwood itself and more subtle differences in preparation of the agar medium.

Non-aggressive subgroup vc tests with a single pairing per plate were incubated at 30℃ in darkness for 8-9 days, and then at room temperature in diffuse daylight, before being assessed after a total of 4-5 weeks. Plates with multiple pairings were incubated for 5-6 days at 30℃ , and assessed after about 2-3 weeks.

2.4 MATING TYPE DETERMINATION

2.4.1 NAN Aggressive

VC tests of unknown isolates against an isolate of the NAN supergroup also determined mating type. Using a standard B mating type supergroup isolate any pairings producing perithecia identified the unknown isolate as A mating type, and those wihout perithecia identified the unknown as B mating type.

The procedure described by Brasier (1981) was used when vc tests had not been carried out. Since *O.ulmi* is hermaphrodite, the fertilisation of protoperithecia of recipient (female) A or B mating type isolates with donor (male) conidia, will result in the production of perithecia if the unknown isolate is of opposite mating type.

Recipient colonies were prepared by centrally inoculating ESA plates with either a known A or B mating type isolate, and incubating the plates at 20 ℃ at least until the agar surface was covered. The recipient colonies were then fertilised in small patches by moving around on their surface a small square (c. 5x5 mm) of agar from a culture of the donor isolate on MEA. This transferred conidia from the donor to the recipient mycelium. An alternative method of fertilisation was to paint small patches of donor conidia onto the recipient colony with 4 mm sable brushes, sterilised by washing in absolute ethanol and rinsed in sterile distilled water. The patches were made in a spiral around the recipient colony, usually about 25 per plate, from a marked starting point and in a known order beginning with control isolates of each mating type. After about 5 days incubation at 20℃ the patches were examined for perithecia and the isolates designated as A or B mating type accordingly. Although unknown isolates should strictly have been tested against known recipients of each mating type, in practice reliable results could be achieved using only an A mating type recipient. NAN isolates readily produce perithecia under these conditions, and the A mating type is especially fertile as the female, recipient parent because of the production of large numbers of protoperithecia on ESA, even in the absence of the B mating type.

2.4.2 Non-Aggressive Subgroup

Due to the lower fecundity of the non-aggressive subgroup mating type tests were not as reliable as with the NAN. In general, the fertilisation methods described above were used, unless prior vc tests had already successfully identified mating type. Despite the fertility

barrier between the subgroups, NAN isolates were usually more reliable as testers because of the large numbers of protoperithecia they produce on ESA. If this still failed to identify mating type, or as an alternative method, non-aggressive isolates were inoculated onto ESA plates together with an NAN isolate of each mating type. The plates were examined for perithecia after appropriate incubation.

2.5 CONTROLLED CROSSES AND ISOLATION OF SINGLE ASCOSPORE PROGENY

Single ascospore progeny from crosses of known male and female parents were obtained using the method described by Brasier & Gibbs (1975a,b), and in more detail by Webber *et al.*, (1986). Plates of ESA were inoculated centrally with the parent chosen to act as female and incubated at 20°C at least until the agar surface was covered. They were then fertilised with conidia of the parent chosen to act as male, applied using a sterile brush as described for mating type determinations. Perithecia developed on further incubation.

A mature perithecium with a sticky ascospore blob at the osteole was picked off using a small diameter tungsten wire loop and rolled around on water agar to remove adhering spores and hyphae. The cleaned perithecium was surface sterilised by dipping in a drop of 2% household bleach followed by 50% ethanol on glass well slides for 20 seconds each, before being rinsed in sterile water. The neck of the perithecium was broken off as near to the bowl as possible using a tungsten needle or watchmakers forceps, and transferred to a fresh 2% MEA plate. Ascospores could usually be seen oozing from the broken neck, although sometimes not until it had been covered with a drop of sterile water. Transfer of the perithecium to one or two more fresh plates for varying periods of time gave a range of spore densities. Perithecia which did not ooze were discarded. After the perithecium had been removed a drop of water was added and the spores spread across the plate. Germlings were picked off after 12-36 hours incubation at 25 °C , usually over a time period of about 12 hours to reduce the bias towards faster growing germlings. Any germlings not obviously originating from a single ascospore were rejected.

Sometimes perithecia from vc test plates were used if it was not essential to have known male and female parents. This was especially useful with non-aggressive subgroup crosses, since advantage could be taken of batches of agar which encouraged the production of large numbers of perithecia.

2.6 SELECTION OF CARBENDAZIM TOLERANT MUTANTS

Tolerance to the fungicide carbendazim or MBC was used as a nuclear marker in investigations of various aspects of vc reactions and interactions between the NAN and non-aggressive subgroups.

Interest in carbendazim tolerance originated when fungicides of the benomyl group, to which carbendazim belongs, were being increasingly used to control Dutch elm disease and it became necessary to assess the threat that tolerance presented to effective control. Brasier & Gibbs (1975a) found that growth of *O.ulmi* was completely inhibited at 0.25 ppm carbendazim, and that tolerant mutants could be selected by spreading 0.1 ml of a dense conidial suspension (c. $5x10^9$ conidia/ml) onto MEA + 0.5 ppm carbendazim (Appendix 1). Most spores produced germ tubes and then ceased growth, but after about 10 days incubation occasional normal looking colonies could be seen, which were then subcultured onto MEA + 0.5 ppm carbendazim. The frequency of tolerant colonies was about 1 per $1.3x10^9$ conidia, and tolerance was shown to be due to nuclear mutation, probably at a single locus, named as *tol*. Further work by Webber *et al.*, (1986) has confirmed this, and identified three different tolerant phenotypes, probably conferred by mutations at the same locus. Webber (1983) has shown that compared to their sensitive counterparts carbendazim tolerant isolates are less pathogenic and slower growing in vitro, and produce smaller lesions when inoculated into the bark of freshly cut elm logs.

Tolerant isolates have been invaluable in various studies where a nuclear marker was required (eg Brasier, 1977, 1983b, 1984; Webber & Brasier, 1984), selected using the method of Brasier & Gibbs, (1975a). This basic selection method has been used whenever carbendazim tolerant isolates were required during the investigations described here.

2.7 SELECTION AND CHARACTERIZATION OF IPRODIONE TOLERANT MUTANTS

A second marker was required for use in conjunction with carbendazim tolerance to facilitate studies of mycelial interactions, particularly non-aggressive subgroup vc reactions and NAN/non-aggressive interactions. Tolerance to the dicarboximide fungicide iprodione was characterized and shown to be suitable for this purpose. An account of this work has been published (Mitchell, 1987) and will not be described in detail here.

Iprodione is fungistatic in its action, allowing sensitive isolates to grow at a reduced rate in its presence. This was found to be a disadvantage in some circumstances. The ED50 for radial growth rate on MEA was about 1.5 ppm for isolates of both aggressive and non-aggressive subgroups, although the ED50 for conidial germination of two NAN isolates was about 19 ppm.

Iprodione tolerant mutants were readily selected by inoculating mycelial plugs onto MEA + 5.0 ppm iprodione (Appendix 1), a concentration which slowed growth sufficiently to allow any tolerant sectors to grow out. Faster growing tolerant sectors were then picked off onto MEA + 5.0 ppm iprodione after about 10 days incubation.

Using the above selection procedure three morphologically distinct classes of iprodione tolerant mutant were recognised. Mutants of all three classes retained their tolerance over at least 15 mass subculturings in the absence of the fungicide, and all were sensitive to carbendazim. More detailed investigation showed that all three classes are probably suitable for use as nuclear markers, although class 1 mutants have been used in preference to classes 1 and 2. Class 1 mutants were found to be significantly less pathogenic ($P<0.01$) than sensitive isolates when inoculated into four-year-old clonal *U.procera*, and to produce smaller lesions than both sensitive and carbendazim tolerant isolates when inoculated into the bark of freshly cut logs of healthy *U.procera*. Dicarboximide tolerant isolates of other fungi isolated from treated crops or fungicide amended media typically show reduced pathogenicity (Beever & Byrde, 1982; Leroux & Fritz, 1984).

Investigation of the inheritance of class 1 iprodione tolerance strongly suggested that tolerance is conferred by mutation at a single locus *(ipr-1)* which was found to be linked to the mating type locus. It is therefore likely that *ipr-1* is also linked to the locus conferring carbendazim tolerance, since this too is linked to mating type (Webber

et al., 1986). The results of crosses between mutants of the three classes indicated that they represent mutations at two or three separate but closely linked loci, *ipr-1*, *ipr-2* and *ipr-3* respectively.

2.8 USE OF FUNGICIDE TOLERANT MARKERS *IN VITRO*

Carbendazim tolerant isolates were used by Brasier (1984) to show that the synnemata formed along either side of the mycelial barrage between two vegetatively incompatible isolates are produced by the opposing isolate (the 'penetration effect'). The depth of penetration, as demonstrated by the bands of synnemata, could then be used both to define and recognise the different types of incompatible reaction.

However, non-aggressive subgroup isolates produce synnemata too sparsely and unpredictably for a reliable assessment of penetration between non-aggressive isolates or non-aggressive and NAN isolates. By opposing two isolates tolerant to carbendazim and iprodione respectively, it was possible to accurately measure penetration of both isolates from the same plate without recourse to synnemetal stab isolations and characterization of the resulting colonies.

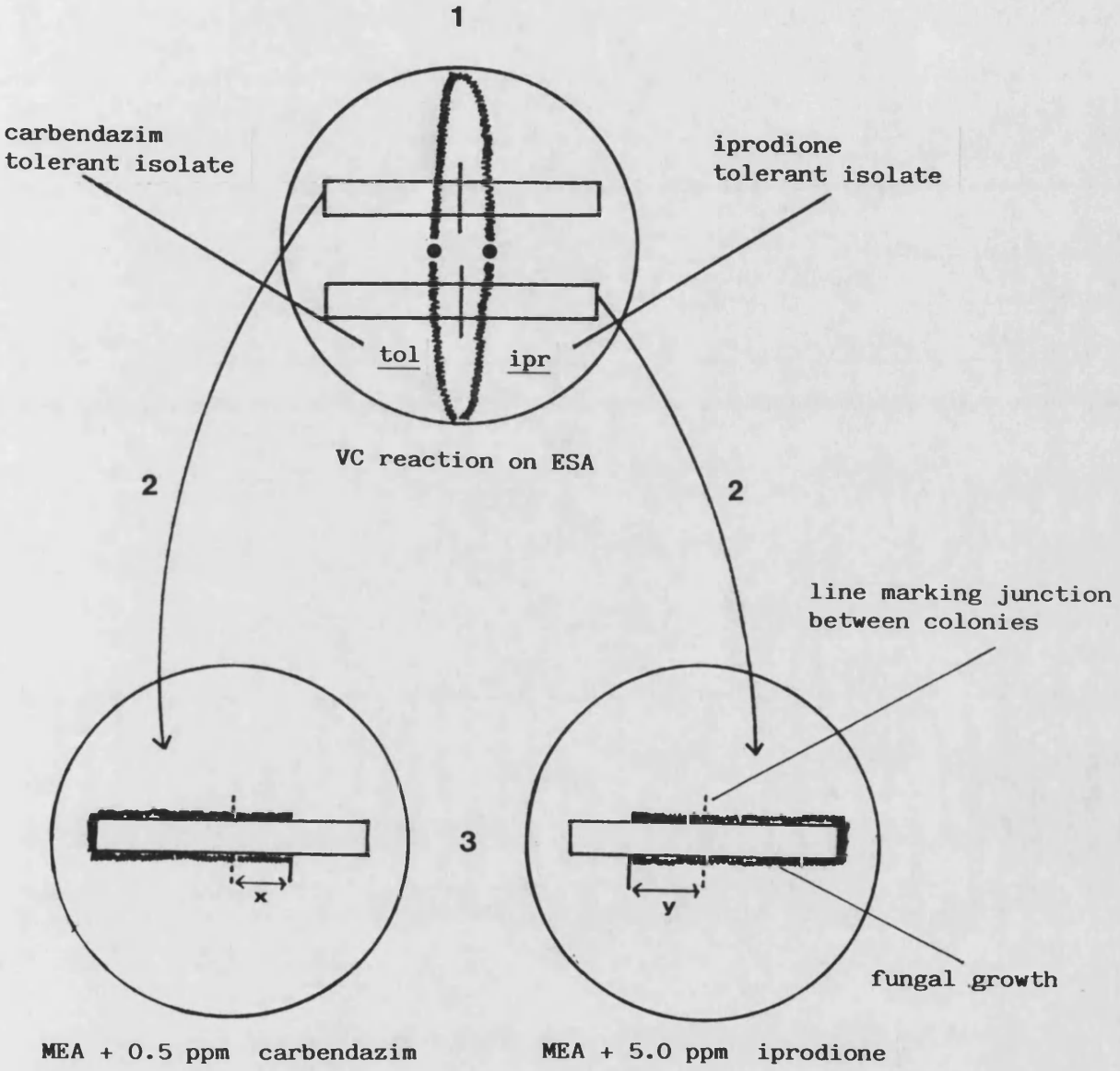
Carbendazim and iprodione tolerant mutants were selected for each isolate to be tested using the procedures described above. Reciprocal combinations of the markers (ie carbendazim tolerant isolate X versus iprodione tolerant isolate Y, and iprodione tolerant isolate X versus carbendazim isolate Y) were used for each pair of isolates tested to reduce any effects that the fungicide tolerant mutations might have on relative penetrating abilty. Since the experiments did not require assessment of the presence of synnemeta and perithecia it was possible to use continuous incubation in darkness.

After an appropriate period of incubation, usually 3-4 weeks, isolations were made from each test plate onto MEA + 0.5 ppm carbendazim and MEA + 5.0 ppm iprodione. In early experiments, strips of agar about 5-10 mm wide were cut along a line perpendicular to the junction between the two colonies and transferred face down onto each of the fungicide agars (Figure 2.1). The junction between the colonies was marked on the re-isolation plates. However, this method of transferring the strips and marking the junction was awkward and inaccurate, and refinements were made to try and overcome these problems.

A 55-60 mm long piece of aluminium comb to which a handle had been attached was used to make the isolations from the test plates. A central mark on the comb could be easily positioned at the junction between the two colonies and then aligned with a mark on the re-isolation plates (Figure 2.2). Several combs were used in sequence to allow them to cool after flaming. After 1-2 days

incubation the extent of penetration of each isolate into the other from the junction was measured from the growth of mycelium along either the agar strips or the inoculations made with the comb.

Figure 2.1 Measurement of Penetration In Vitro Using Nuclear Markers - Strip Re-Isolation Method

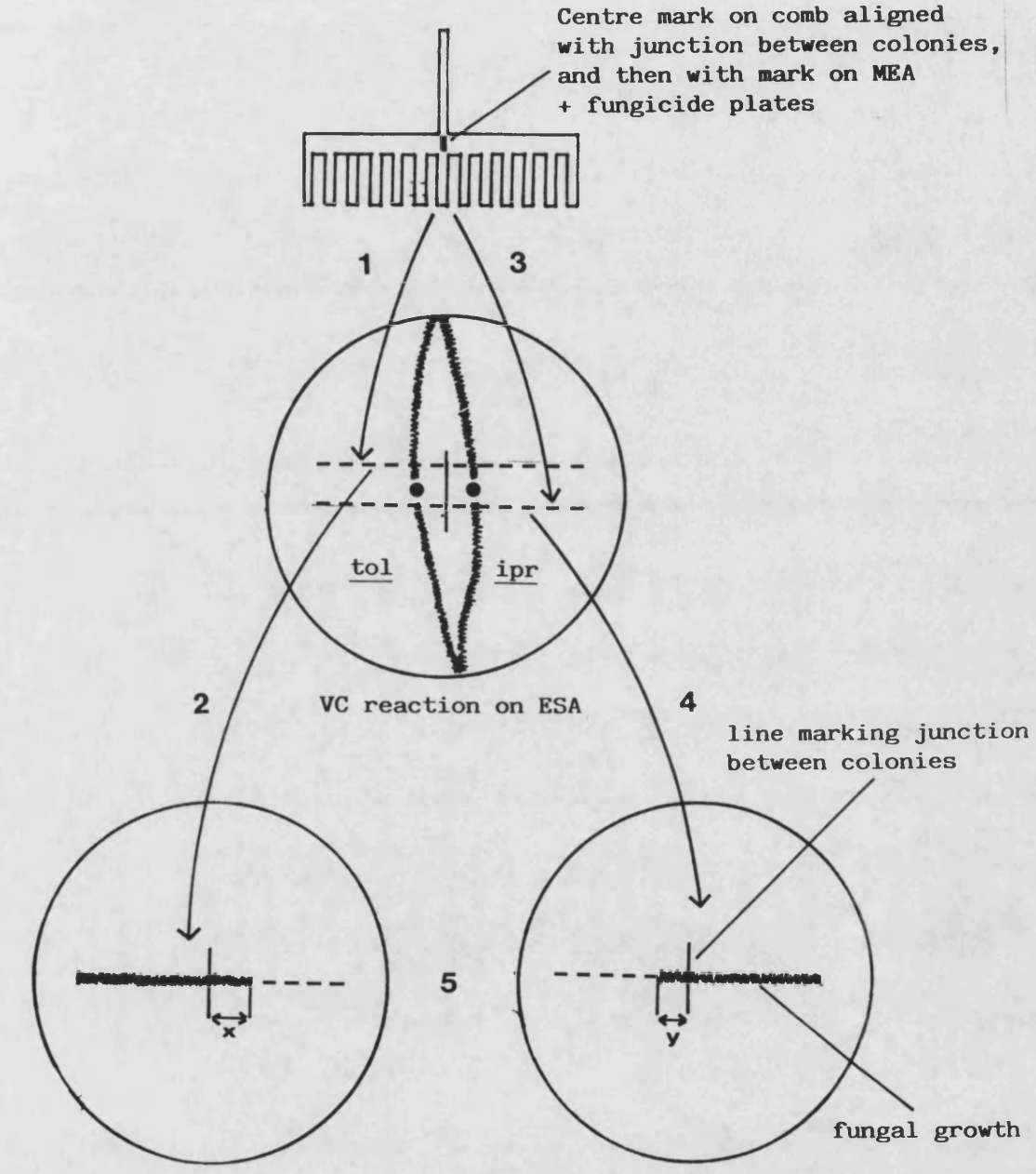


1 Strips cut across vc reaction between fungicide tolerant isolates

2 Strips transferred face down to fungicide-amended agar plates

3 Penetration into each isolate (x and y) measured from growth of tolerant mycelium after 1-2 days incubation

Figure 2.2 Measurement of Penetration in Vitro Using Nuclear Markers - Comb Re-Isolation Method



1 Flamed comb placed across vc reaction between fungicide tolerant isolates

2 Inoculum transferred to MEA + carbendazim plate

3 Comb flamed again and placed across vc reaction

4 Inoculum transferred to MEA + iprodione plate

5 Penetration into each isolate (x and y) measured from growth of tolerant mycelium after 1-2 days incubation

2.9 INOCULATION METHOD FOR EXPERIMENTS IN BARK

Experiments involving the measurement of lesions in elm bark all used the same basic method, described by Webber (1979) and Webber & Hedger (1986).

Freshly cut logs of healthy *U.procera* about 1m in length and 0.2-0.3 m in diameter were sealed at the ends using Lac Balsam, a non-fungicidal tree wound sealant, to prevent the bark drying out and separating from the sapwood. To limit colonisation by other fungi the ends were sealed as soon as possible after felling, and the bark usually inoculated within 1-2 weeks.

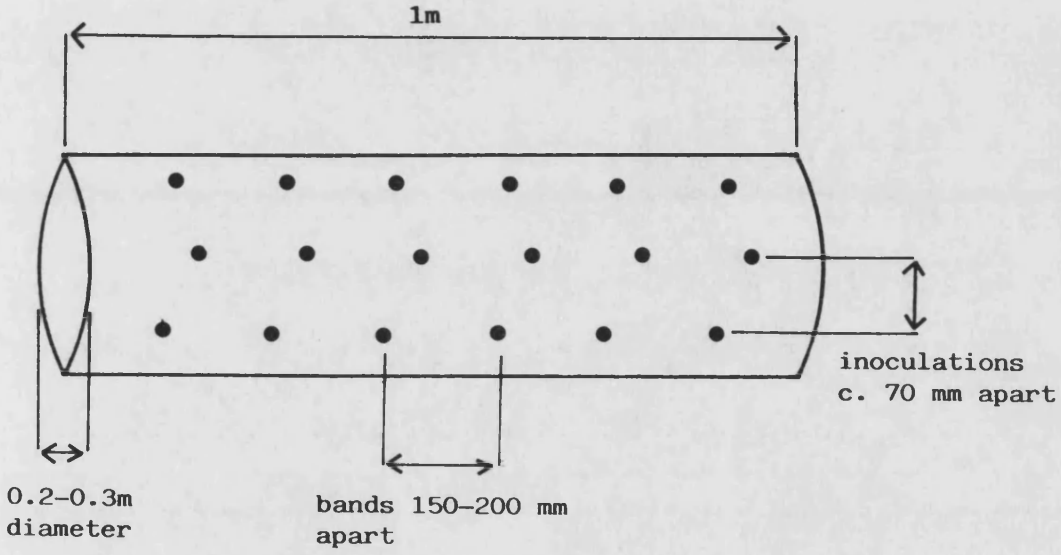
Inoculations were made in bands around the circumference 150-200 mm apart, with the inoculations in each band about 70 mm apart, giving 7-12 inoculations per band and 4-7 bands per log (Figure 2.3). The pattern and spacing of the inoculations was sometimes varied according to the number of logs available and the requirements of individual experiments. In any one experiment all the logs used were from the same tree.

Two drops of a dense spore suspension (about $1x10^7$ spores/ml) produced by 3-5 days growth in shake cultures of Tchernoffs medium (Appendix 1), were introduced from a syringe into holes made in the bark using a sterile 5 mm diameter cork borer. All of the inner bark was removed and usually separated easily from the sapwood at the cambium, but there was inevitably some damage to the sapwood. After the spore suspension had been dropped into the hole the bark core was replaced and sealed with PVC tape, which was then secured with staples and labelled.

The logs were incubated either in a cellar at about 17-18°C or at about 27-29°C , and rotated a quarter turn each week. Extra inoculation points were made so that lesion development could be followed and the experiment assessed after an appropriate period of incubation.

Typical lens-shaped lesions were formed (Lea, 1977; Webber, 1979; Webber & Hedger, 1986), with the greatest extent in the inner bark slightly away from the cambium. A typical lesion is shown in Plate 2.4. Lesion areas were calculated from the weights of cut out tracings of the lesions at their maximum extent.

Figure 2.3 Standard Log Inoculation Pattern for Experiments in Bark



● inoculation point

RESULTS

SECTION I INVESTIGATION OF THE SAPROTROPHIC PHASE OF THE NAN AGGRESSIVE SUBGROUP OF *O.ULMI*

In order to study the interaction between the aggressive and non-aggressive subgroups, some further information was needed on aspects of the population behaviour of each subgroup alone, particularly during the saprotrophic phase. Appropriate studies on the non-aggressive subgroup were not possible in 1983 when the work was initiated, owing to the virtual disappearance of this form of the pathogen in Britain since the late 1970s. Such studies were undertaken later in Spain (see Section II). A detailed investigation was therefore carried out on aspects of the saprotrophic phase of the NAN aggressive, together with comparative studies on the pathogenic phase. Since mature English elms have been killed over much of southern England, the work was largely carried out on a surviving population of smooth leaved elm in East Anglia.

The results of this work have for convenience been separated into into three topic areas, although they obviously represent closely interrelated parts of the same investigation. These areas are:

1. The establishment and subsequent population structure of the pathogen in the saprotrophic phase.

2. Comparison of the structure of saprotrophic and pathogenic phase populations.

3. The occurrence of the penetration effect between isolates opposed *in vivo*.

3 THE ESTABLISHMENT AND POPULATION STRUCTURE OF THE SAPROTROPHIC PHASE

3.1 INTRODUCTION

The work of Lea (1977) was the first to indicate that considerable genetic diversity exists in saprotrophic phase populations of *O.ulmi*. Lea showed that in bark a mosaic of morphologically and therefore probably genetically different types occupied discrete territories in an area of only a few hundred sq cm. Subsequently, Brasier (1984, 1986a) used the fungus' vegetative incompatibility system to show that the pathogenic phase of the NAN subgroup often comprises a uniform component - the vc supergroup - and a heterogeneous component of diverse vc groups. It was therefore likely that the morphological and implied genetic diversity among isolates from bark described by Lea was also maintained by the vegetative incompatibility system.

The mosaic pattern observed by Lea in bark fully colonised by vector beetles and *O.ulmi* is likely to have been influenced by a number of factors during the initial establishment of *O.ulmi* in dying bark, in particular: the proportion of maternal galleries of the vector beetles contaminated with *O.ulmi*, the number of genotypes introduced to each gallery, the number of genotypes that become established in each gallery, and the extent of colonisation of the bark by genotypes from the underlying xylem (ie feedback from the pathogenic phase).

Two distinct periods of bark colonisation can be recognised, based on the involvement of *O.ulmi*'s pathogenic ability (cf Webber & Hedger, 1986). In the first - the 'expanding gallery period' - pathogenic ability is likely to be involved in the colonisation of bark in association with larval galleries expanding into otherwise still living bark with some degree of host resistance. The fungus will be acting saprotrophically in the second longer period, when adjacent gallery systems and associated fungal lesions have met to more or less fully occupy the bark, and there is no longer any living bark tissue. The latter will be referred to as the 'fully colonised' period.

There is good evidence from the work of Lea (1977) and Webber & Brasier (1984) that considerable changes take place during the saprotrophic phase (Chapter 1.2.4), and that the structure of the *O.ulmi* population represented in the sporeloads of the subsequent generation of beetles depends on these changes. The changes are

thought to be the result of interactions between different mycelia, and the periods of recolonisation which probably take place from late summer to late spring of the following year (Brasier, 1984). Feedback from the pathogenic phase also plays an important role, both as increasing colonisation of the bark by pathogenic phase isolates from autumn to early summer of the following year, and through recombining the pathogenic and bark phases (Webber & Brasier, 1984). It is possible that any changes in population structure could be detected as differences between the population present in autumn following the initial establishment period, and the population present in late spring just before beetle emergence. A prerequisite of any investigation of population structure is a simple and reliable method for identifying different genotypes. Previous work with *O.ulmi* suggested that the fungus' vegetative incompatibility system could be used not only to provide the necessary means of genotype indentification, but would also facilitate analysis of the dynamic aspects of bark colonisation, such as:

i. The initial colonisation of expanding galleries.

ii. The establishment of the mosaic as galleries and associated fungal lesions meet.

iii. The extent of feedback from the pathogenic phase.

iv. The temporal development of the mosaic.

There are however certain conceptual problems in the use of the vc system for this kind of population analysis, particularly regarding the status of the large number of isolations involved. This is an aspect of the problem of defining a fungal individual.

Since fungal populations are now widely recognised as being made up of groups of vegetatively incompatible isolates, and the inherent characteristics of vc itself tend to maintain genetic integrity, it is reasonable in many cases to consider a single vc group as an individual (Cooke & Rayner, 1984). However, in the large number of species where extensive vegetative growth or the production of conidia results in spatially separated but genetically identical mycelia, two isolates may be completely isogenic but it is still necessary to recognise that they have been sampled from separated mycelia. It is also important to make a distinction between such isolations from genetically identical but spatially separated mycelia and different isolations of a continuous mycelium recently developed from the same inoculum, even if its continuity can only be assumed.

The situation becomes further complicated if there is little genetic exchange between different vc groups, perhaps due to the absence of a sexual stage. Many generations of asexual reproduction could then result in small but accumulating genetic differences between isolates of the same vc group. In this case, although there are some genetic differences between isolates of the same vc group, they will still be more similar to each other than they are to isolates of other vc groups. This seems to be the situation in some Aspergillus species (Croft & Jinks, 1977), where different vc groups may be evolving into sibling species through accumulating mutations (Croft & Dales, 1984). Isolates of the same vc group but with different nuclear genetic backgrounds can be characterized by a variety of other means, such as colony morphology, growth rate, isozyme variation and so on.

In order to clarify the terminology that has been used during the course of the present investigation based on the use of vc differences, it is necessary at this stage to make some assumptions of the results. Pathogenic phase isolates of *O.ulmi* taken from different diseased trees, each the result of a separate beetle infection, are clearly derived from spatially separated mycelia. Subsequent vc tests may show some isolates to be of the same genotype, or more strictly of the same vc genotype, ie isogenic at all vc loci. The interpretation of results of saprotrophic phase sampling is more complex, since adjacent isolations may often be from the same mycelium. Therefore, samples taken from fully colonised bark in order to analyse the mosaic structure will result initially in a series of isolations which, after vc testing, might be grouped as different genotypes occupying discrete areas. Similarly, a series of isolations could be made from a discrete expanding gallery, and vc tested to determine the number of vc genotypes present. Further analysis may show the same genotype to be present in different galleries.

In *O.ulmi* the great diversity within the population and the continual input of recombination from sexual reproduction means that the large majority of pathogenic and saprotrophic phase isolates defined after initial vc analysis, with the main exception of the NAN and EAN supergroups, will be different vc groups or individuals. Therefore, since most isolates are effectively unique vc genotypes it is perhaps misleading to describe them as vc groups, and may be more accurately refered to as vc types.

3.2 OBJECTIVES

The objectives of this part of the work can be summarised as:

1. To investigate the early stages of bark colonisation, in particular the proportion of beetle galleries contaminated with *O.ulmi*, the number of vc types present in such galleries, and the stage at which pathogenic phase isolates from the xylem begin to colonise the bark.

2. To verify the predicted mosaic pattern in fully colonised bark using the fungus' vegetative incompatibility system.

3.3 MATERIALS AND METHODS

3.3.1 Field Sites

Most of the sampling was carried out on Mersea Island, Essex (NG reference TM 01), in particular at Barrow Hill Farm (NG reference TM 022 144). This site was especially suitable because of small fields with almost 4 km of elm hedges in an area of only 75 ha (Plate 3.1).

The elm population of Mersea Island and the surrounding areas of East Anglia is predominantly smooth leaved elm. Richens (1967) has suggested that the people associated with the Red Hills salt workings found in the area introduced these elms from northwest France shortly before the Roman occupation. Elm is an important tree on the island, making up the great majority of hedges in an area which is largely agricultural land apart from the town of West Mersea, rising to only 21m above sea level and otherwise poorly protected from the North Sea.

The NAN aggressive has been present in the area since the early 1970s, probably spreading originally from Tilbury docks, one of the ports through which the NAN is thought to have entered Britain and only some 55 km away to the southwest. In contrast to nearby English elm areas, the rate of progress of the current epidemic in smooth leaved elm has been relatively slow (Gibbs, 1978a), and consequently many healthy and recently diseased trees remain in the area.

Some saprotrophic phase samples were also obtained from a diseased wych elm in Alton, Hampshire (NG reference SU 704 389). English elm is the most frequent elm in this area, now largely reduced to sucker regeneration following the epidemic of the 1970s. Many of the local wych elm survived into the early 1980s probably due to the feeding preference of elm bark beetles for English rather than wych elm (Webber & Kirby, 1983), but most have now succumbed.

3.3.2 Sampling and Isolation Methods

Bark slabs and samples of the underlying xylem were taken from diseased trees and the approximate positions of sampled trees recorded on a 1:25000 scale map of Mersea Island, or a 1:5000 scale plan of the Barrow Hill Farm site drawn from the map. Isolations were made using either the chip or dilution method as described in Chapter 2.1.2. Most used the chip method since it was faster and less complicated than the dilution method, and it would otherwise have been impractical to isolate from a sufficiently large number of galleries. The dilution method was used to check that the results were not seriously biased by the technique, since in a more detailed comparison Lea (1977) suggested

Plate 3.1 Hedgerow of Smooth Leaved Elm at Barrow Hill Farm

that the chip method tended to isolate from mycelia, and the dilution method from spores. Similarly, soil dilution plates tend to favour isolations from spore sources (Warcup, 1955).

A limited number of bark isolations were made in autumn 1983, and provided useful preliminary data. In summer 1984 a detailed series of isolations was made from expanding beetle galleries up to the stage at which larval galleries from adjacent maternal galleries and associated fungal lesions began to meet. Each expanding gallery system was assigned to one of four stages according to its state of development (Figure 3.1):

Stage 1. Entrance holes reaching the inner bark/xylem interface but with limited excavation of maternal galleries.

Stage 2. Maternal galleries, mostly with eggs and recently hatched larvae, and sometimes very short larval galleries.

Stage 3. Maternal galleries with short larval galleries up to about 15-20 mm long.

Stage 4. Maternal galleries with larval galleries longer than about 20 mm but still more or less discrete in otherwise still living and uncolonised bark.

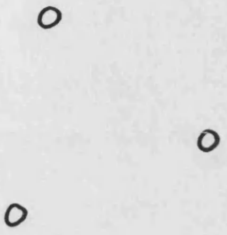
It should be noted that the above classifications are artificial divisions of a process of continuous gallery development.

The beetle species making the gallery (either *S.scolytus* or *S.multistriatus*) was sometimes confirmed by the presence of an adult within the maternal gallery, or was judged from the size of the gallery. With experience, galleries of each species could be distinguished with a high degree of confidence.

Pieces of the xylem underlying each bark sample were also removed to allow isolations to be made from the associated pathogenic phase. A complete horizontal section was taken, usually from beneath the middle of the bark slab, and assumed to be representative of the distribution of *O.ulmi* in the xylem over the whole vertical height of the sample. The distribution and intensity of the xylem streaking was recorded in the laboratory, and isolations made from freshly exposed sapwood of the current annual ring following surface sterilisation with industrial methylated spirits. For the Alton wych elm samples it was possible to isolate from around the whole circumference of the trunk, although the orientation of the bark samples to the xylem isolations was not recorded.

Isolations from fully colonised bark were made in autumn 1983

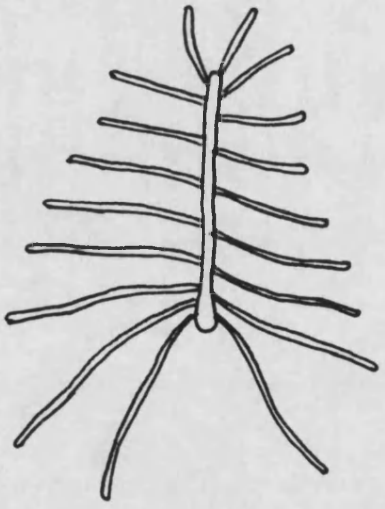
Figure 3.1 Developmental Stages of Expanding Galleries



STAGE 1
Entrance holes

STAGE 2
Maternal galleries with eggs and recently hatched larvae

STAGE 3
Maternal galleries with short larval galleries up to 15–20 mm long



STAGE 4
Maternal galleries with larval galleries longer than 20 mm

Actual size

and spring 1985 using the grid system described in Chapter 2.1.2. The spring 1985 sampling was therefore of the same beetle generation as that initiating the expanding galleries sampled in summer 1984.

3.3.3 Analysis of Isolations

i. Expanding Galleries:

Multiple isolations from each single discrete gallery were vc tested in 4x4 patterns on ESA (Chapter 2.3.1) to resolve them into different vc types. If the number of vc types was small then a single test was usually sufficient, but further tests were often necessary in more complex situations. Once the isolations from a gallery had been fully analysed each vc type was given a code taken from the lowest representative isolation number. In some cases all vc types from a particular bark sample were tested against pathogenic phase vc types isolated from the underlying xylem.

ii. Fully Colonised Bark:

Isolations from substantial bark grids involving many gallery systems were analysed in a series of vc tests, initially in overlapping groups of 16 in 4x4 patterns (Chapter 2.3.1). Eventually all the vc types in each grid occupying more than about 5 sq cm were tested in virtually all combinations. The remaining vc types were tested in most combinations within their immediate area and against all neighbouring vc types occupying relatively large areas. Once resolved into vc types tests were in some cases made against pathogenic phase vc types isolated from the xylem.

3.4 RESULTS

3.4.1 Comparison of the Alternative Isolation Methods

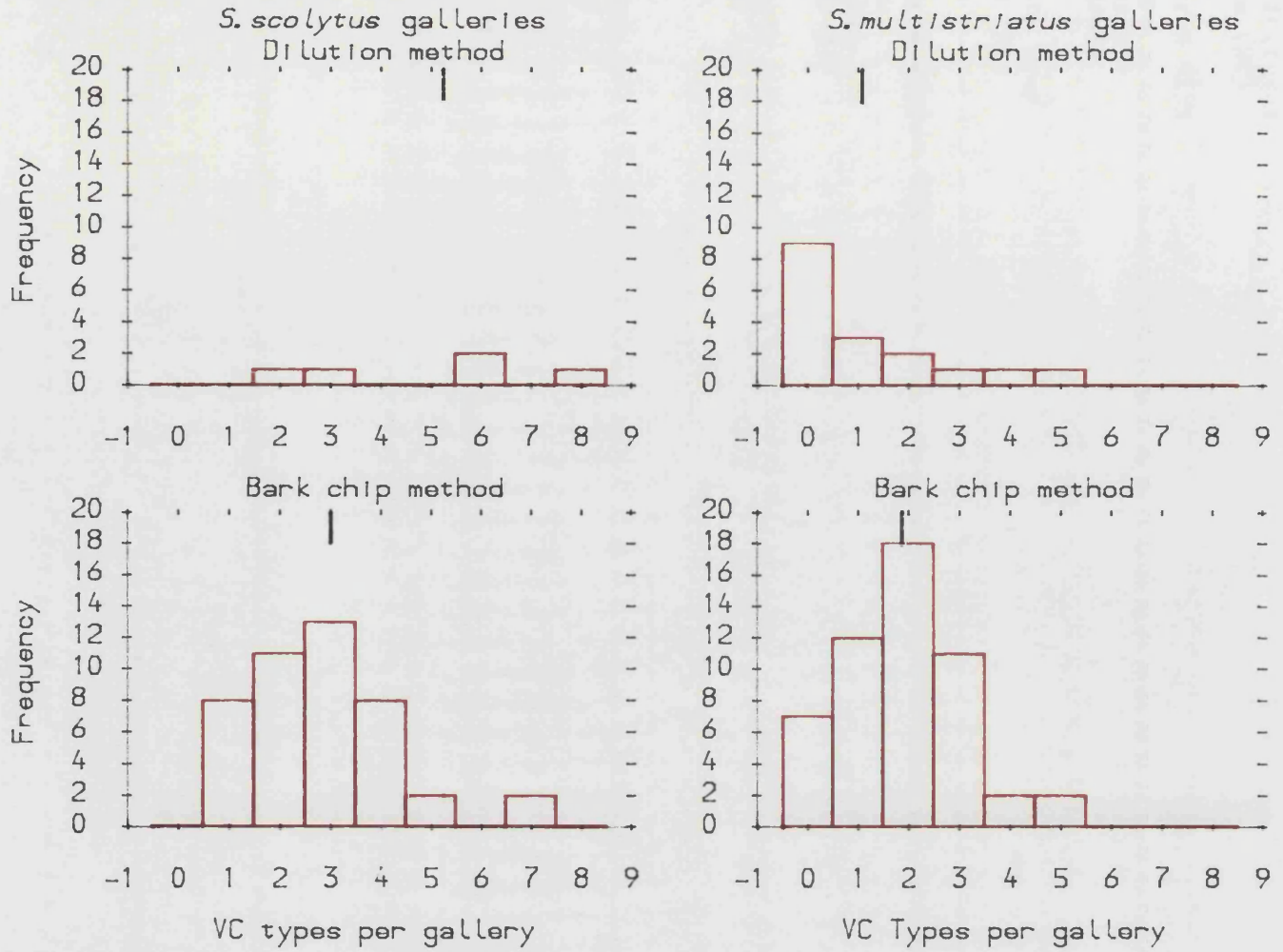
Data for the number of isolates per gallery for each beetle species obtained using the bark chip and dilution methods were pooled and compared (Figure 3.2). The relatively small number of galleries for which the dilution method was used did not allow comparison with the chip method at each of the four stages of expanding galleries. The results for isolations from *S. multistriatus* galleries were analysed using a 2x2 contingency table chi squared test. The two classes consisted of galleries from which no *O. ulmi* was isolated, and galleries with at least one vc type. A significantly higher proportion of galleries failed to give *O. ulmi* using the dilution method ($P<0.01$). The number of *S. scolytus* galleries sampled using the dilution method was too small for statistical analysis.

3.4.2 The Establishment of *O. ulmi* in Bark

The prolonged period of hot, dry weather during the summer of 1983 made it difficult to find bark samples suitable for investigating establishment. This was probably due to intense competition for breeding material among an increased beetle population resulting from a large second generation. Many beetles were forced to attempt gallery excavation in bark with enough sap activity to prevent either successful excavation and the laying of eggs or the development of larvae. However, this provided an opportunity to isolate from discrete, failed galleries in the autumn when galleries would otherwise have more or less fully occupied the bark. In the following season, although such bark became more suitable for beetle colonisation, the old failed galleries had to be recognised when making isolations. When the beetles began excavating galleries in 1984, *O. ulmi* was apparently still confined to limited lesions around the failed galleries, and as the new larval galleries developed they turned away from these lesions. Presumably at a later stage this earlier inoculum of *O. ulmi* became an integral part of the bark population.

The location and status of the bark samples involved are given in Table 3.1. The 1983 isolations were made from bark samples from Haycocks (HAY), Waldegraves Farm (WF) and Barrow Hill Farm (BHF-1). The small number of discrete galleries from which isolations were made indicated that more than one vc type was present in most galleries. Detailed comparative isolations from expanding gallery stages 1-4 were carried out in 1984 using Barrow Hill Farm samples 2, 3, 4

Figure 3.2 Comparison of Bark Chip and Dilution Method Isolations from Expanding Galleries



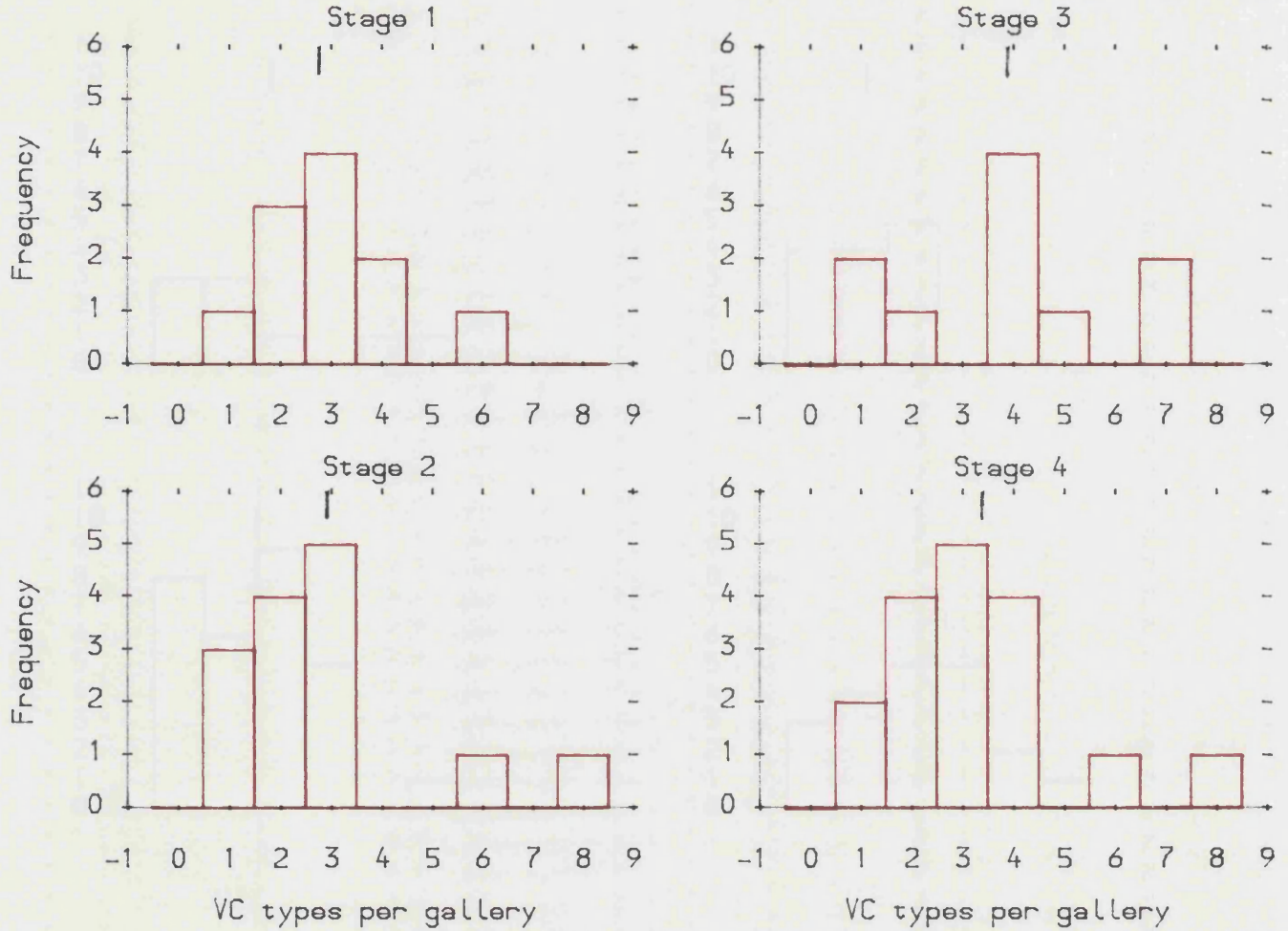
Contingency table analysis for isolations from *S. multistriatus* galleries showed that the proportion of galleries from which no *O. ulmi* was isolated was significantly higher for the dilution method ($P<0.01$). Means indicated by vertical lines.

Table 3.1 Location and Status of Bark Samples

Sample*	Date	Elm species	Stage of colonisation -
Haycocks (HAY)	22/7/83	U. carpinifolia	Mostly S. multistriatus expanding galleries.
Waldegraves Farm (WF)	12/10/83	" "	Mostly failed breeding of both beetle species.
Barrow Hill Farm Sample 1 (BHF-1)	20/10/83	" "	Mostly failed breeding of both beetle species, with some fully colonised bark.
Alton wych elm (AL)	31/10/83	U. glabra	Mostly fully colonised by S. multistriatus, with some failed breeding.
Barrow Hill Farm Sample 2 (BHF-2)	12/7/84	U. carpinifolia	Expanding galleries of both beetle species.
Sample 3 (BHF-3)	12/7/84	" "	Mostly S. scolytus expanding galleries. Taken from directly below BHF-2.
Sample 4 (BHF-4)	25/7/84	" "	Expanding galleries of both beetle species.
Sample 5 (BHF-5)	31/7/84	" "	As BHF-4. Taken from directly above BHF-4.
Trap Log 1 Sample A (TL1-A)	14/8/84	U. procera	Mostly expanding S. multistriatus galleries.
Sample B (TL1-B)	22/8/84	" "	Mostly expanding S. multistriatus galleries.
Sample C (TL1-C)	27/8/84	" "	Mostly expanding S. multistriatus galleries.
Barrow Hill Farm Sample 6 (BHF-6)	21/5/85	U. carpinifolia	Fully colonised, mostly by S. multistriatus, but the few S. scolytus galleries occupying relatively large areas. Grid isolations. From same tree as BHF-4 and 5.

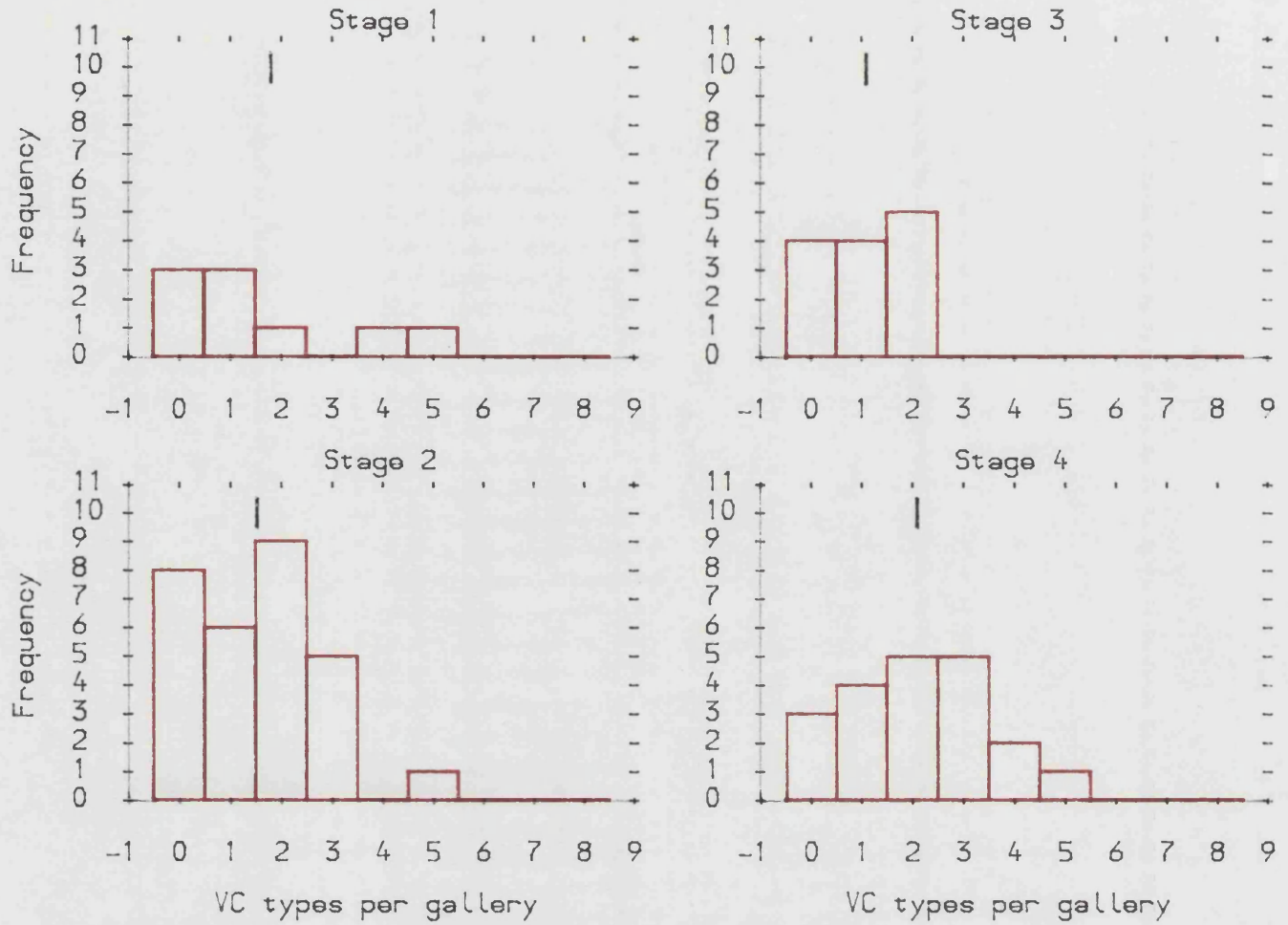
* All from Mersea Island except Alton wych elm samples.

Figure 3.3 Number of VC Types per Gallery in Stages 1-4 of Expanding *S. scolytus* Galleries



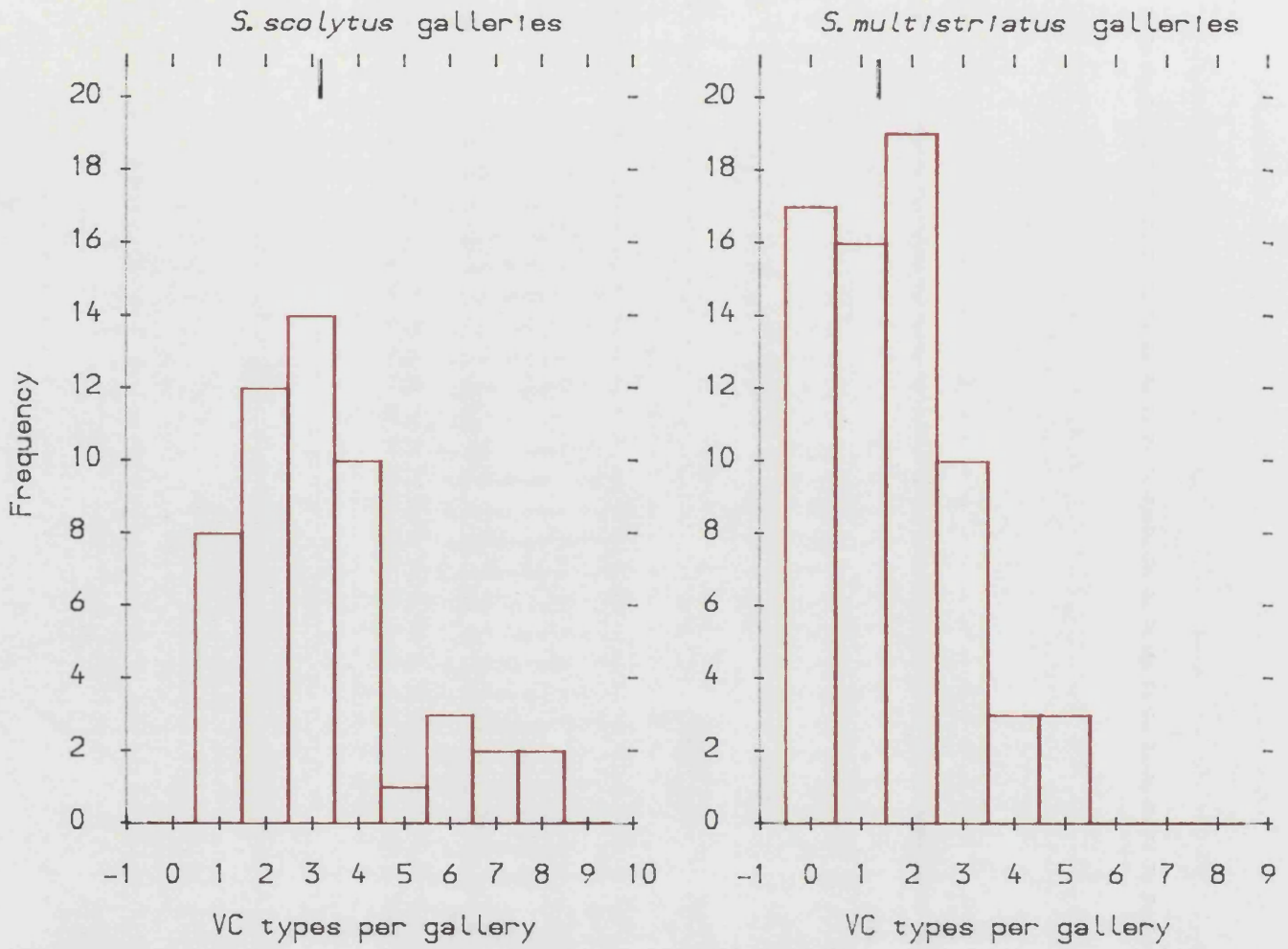
Contingency table analysis showed no significant differences in the mean number of vc types per gallery at each stage. Means indicated by vertical lines.

Figure 3.4 Number of VC Types per Gallery in Stages 1-4 of Expanding *S. multistriatus* Galleries



Contingency table analysis showed no significant differences in the mean number of vc types per gallery at each stage. Means indicated by vertical lines.

Figure 3.5 Number of VC Types per Gallery in Expanding *S. scolytus* and *S. multistriatus* Galleries



Contingency table analysis showed significant differences in the mean number of vc types per gallery ($P<0.001$). Means indicated by vertical lines.

Table 3.2 Mean Number of Bark Chip Isolations per Gallery from S. scolytus and S. multistriatus Stage 1-4 Expanding Galleries

Expanding gallery stage*	Mean no. of bark chip isolations per gallery	
	S. scolytus	S. multistriatus
Stage 1	5	5
2	10	6
3	16	8
4	28	14

* See text for definition and Figure 3.1 for illustration of expanding gallery stages 1-4.

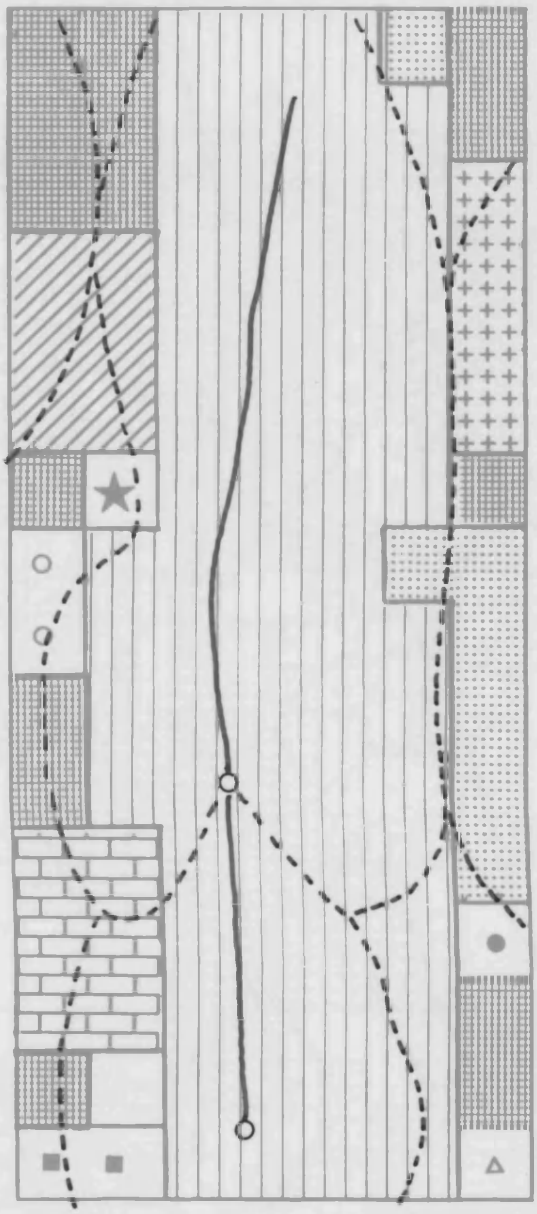
and 5 (BHF-2,3,4 and 5), and from Trap Log 1 (TL1) which had been left in a hedgerow at Barrow Hill Farm and later brought back to Alice Holt Research Station. The results for the mean number of vc types per gallery at each stage are summarised for *S.scolytus* and *S.multistriatus* in Figures 3.3 and 3.4 respectively.

Chi squared analysis of contingency tables showed no significant difference in the mean number of vc types per gallery at each stage of gallery development with either beetle species. However, the pooled data for the different stages (Figure 3.5) showed a highly significant difference between the beetle species. *S.scolytus* galleries had a higher mean number of vc types per gallery than *S.multistriatus* galleries, with means of 3.2 and 1.6 vc types per gallery respectively. Most of the difference lies in the smaller proportion of *S.multistriatus* galleries from which *O.ulmi* was isolated, ie 75% of *S.multistriatus* galleries compared to 100% of *S.scolytus* galleries. The mean total number of bark chips (ie including chips from which no *O.ulmi* was isolated) taken per gallery for each gallery stage and beetle species is shown in Table 3.2. *O.ulmi* from the pathogenic phase was isolated from two out of 16 chips taken from the xylem below bark sample BHF-2, both of the which gave the same vc type. Since bark sample BHF-3 was taken from the same tree as BHF-2 and also directly below, it was assumed that the underlying xylem streaking was continuous. The pathogenic phase vc type was tested against representatives of each of the different vc types obtained from expanding beetle galleries in bark samples BHF-2 and 3. Three (3.5%) of the 88 vc types from the 38 galleries in these two bark samples were found to be compatible with the vc type from the xylem. This was assumed to represent feedback from the pathogenic phase to the bark phase, and consequently the potential for pathogenic phase genotypes to contribute to the sporeloads of the next generation of beetles.

3.4.3 Population Structure in Fully Colonised Bark

Representative bark grids showing the distribution of vc types in fully colonised bark are illustrated in Figures 3.6 and 3.7. The pattern of colonisation in Alton wych elm grid E (AL-E) sampled in October 1983 provides an interesting contrast to that in BHF-6, sampled in May 1985. The BHF-6 isolations were made principally to allow comparison with the expanding galleries sampled in summer 1984. Most of the galleries within the AL-E grid were of *S.multistriatus*. Sample BHF-6

Figure 3.6 Mosaic of VC Types in Alton Wych Elm Bark Sample E



Isolations made from a 10 mm interval grid. Each isolation has been assumed to occupy an area of $1cm^2$.

Each shading pattern or symbol represents a different vc type.
No O.ulmi isolated from areas without shading or symbols.

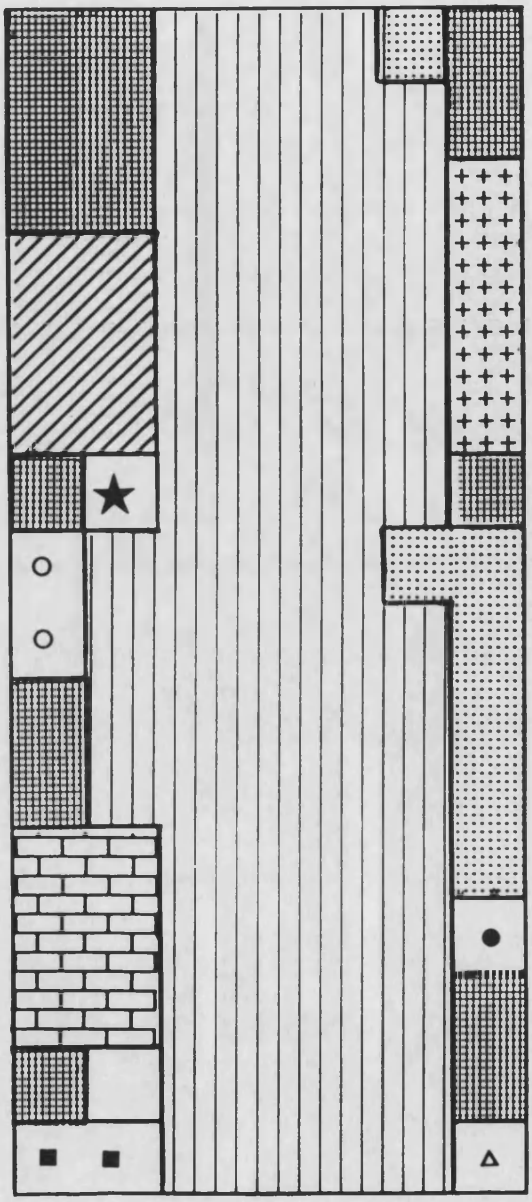
▦ AL-E8 vc type. Compatible with a pathogenic phase vc type isolated from the xylem.

++ AL-E1 vc type. Compatible with a second pathogenic phase
++ vc type isolated from the xylem.

The overlay shows the positions of maternal galleries (solid lines), entrance holes (circles), and extent of associated larval galleries (dashed lines).

Actual size

Figure 3.6 Mosaic of VC Types in Alton Wych Elm Bark Sample E



Isolations made from a 10 mm interval grid. Each isolation has been assumed to occupy an area of $1cm^2$.

Each shading pattern or symbol represents a different vc type.
No O.ulmi isolated from areas without shading or symbols.

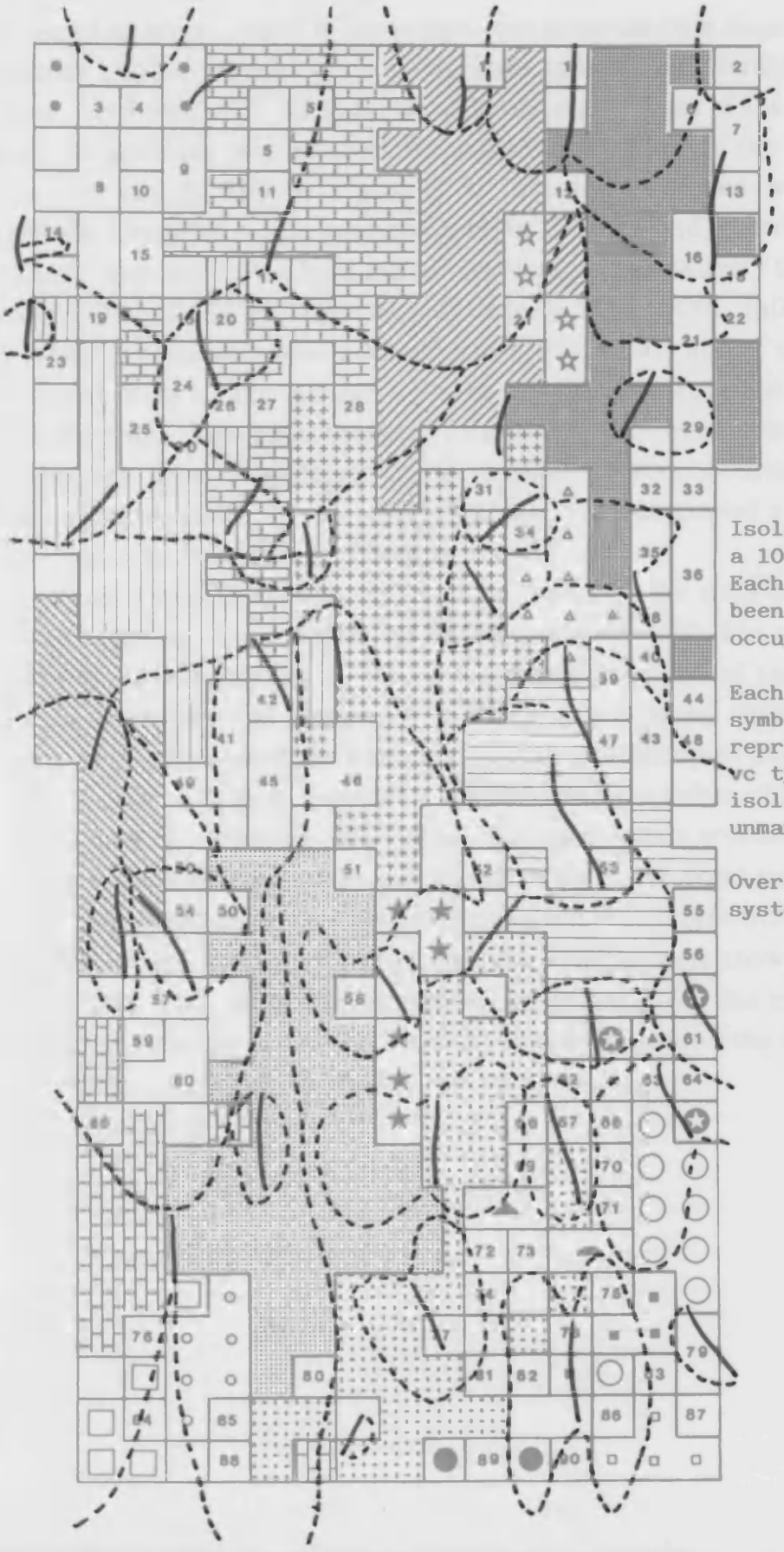
▦ AL-E8 vc type. Compatible with a pathogenic phase vc type isolated from the xylem.

++ AL-E1 vc type. Compatible with a second pathogenic phase ++ vc type isolated from the xylem.

The overlay shows the positions of maternal galleries (solid lines), entrance holes (circles), and extent of associated larval galleries (dashed lines).

Actual size

Figure 3.7 Mosaic of VC types in Barrow Hill Farm Bark Sample 6

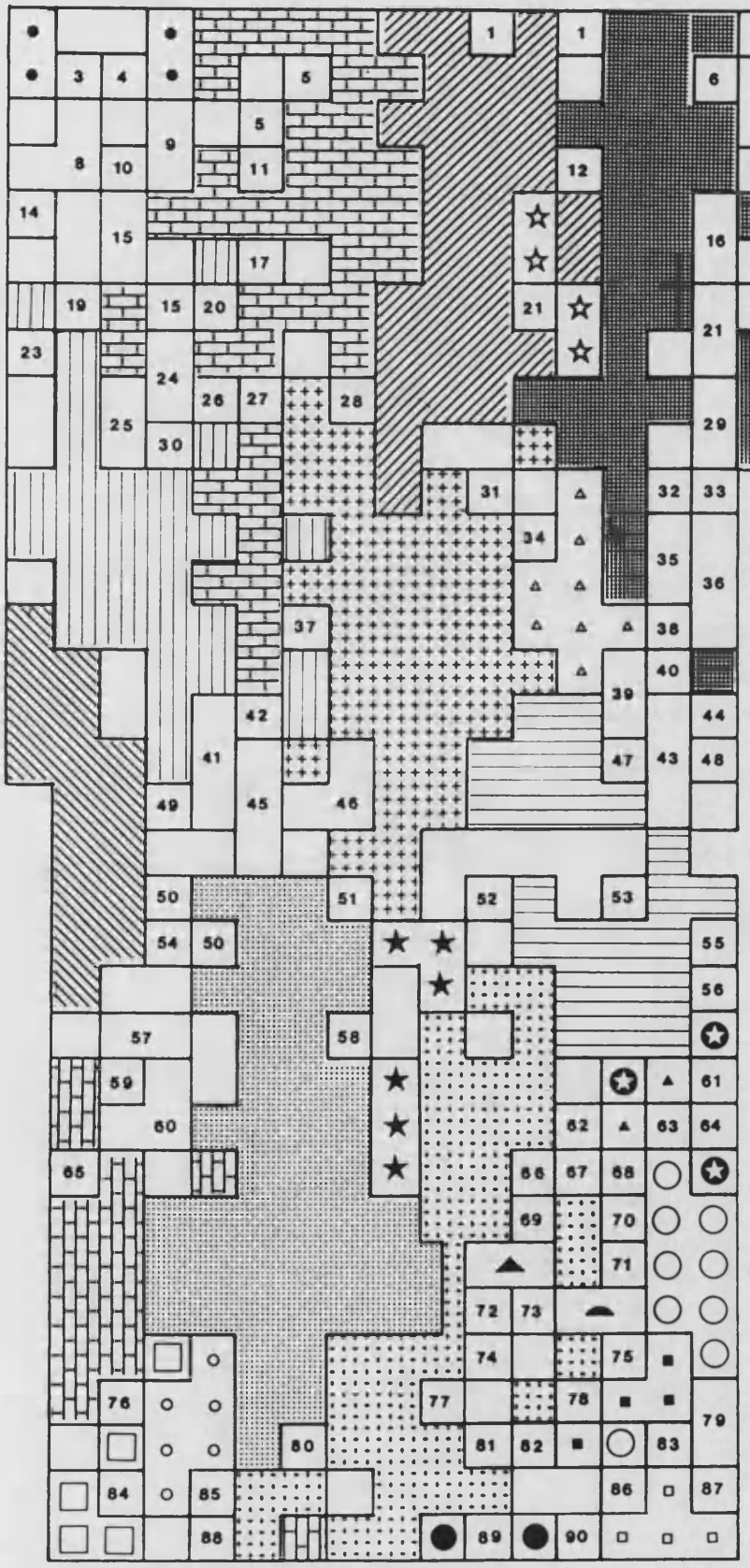


Isolations made from a 10 mm interval grid. Each isolation has been assumed to occupy an area of $1cm^2$.

Each shading pattern, symbol or number represents a different vc type. No O.ulmi isolated from unmarked areas.

Overlay shows gallery systems, as for Fig 3.6

Figure 3.7 Mosaic of VC types in Barrow Hill Farm Bark Sample 6



Isolations made from a 10 mm interval grid. Each isolation has been assumed to occupy an area of $1cm^2$.

Each shading pattern, symbol or number represents a different vc type. No O.ulmi isolated from unmarked areas.

Overlay shows gallery systems, as for Fig 3.6

contained eight *S.scolytus* galleries occupying disproportionately large parts of the grid, and 33 *S.multistriatus* galleries.

In sample AL-E (Figure 3.6) there was some correlation between the positions of the gallery systems and the areas occupied by different vc types. Most of the sample was occupied by a single vc type centred on the position of the two main galleries. Different vc types were associated with larval galleries at the edges of the grid, and two probably originated from the pathogenic phase in the xylem.

In sample BHF-6 (Figure 3.7) the areas occupied were typically elongated, following the grain of the bark and underlying xylem, seen most clearly in the isolates occupying relatively large areas. Out of 536 attempted isolations only 53, or 10%, failed to give *O.ulmi*. The isolates were resolved into 114 vc types, and of these 13 occupied most of the sample, with the remainder represented by a few isolates only. The areas occupied by most vc types were more or less dissociated from the gallery systems. Several vc types occupied discontinuous areas, eg vc types 5 and 68, which occupied a main large area with additional outlying 'islands'.

The frequencies of the different vc types and the areas occupied by each vc type in sample BHF-6 are shown in Figure 3.8. The vc types that were represented by >5 isolations accounted for 12% of the total number vc types and occupied 62% of the area; whereas 61% of the vc types were represented by a single isolation, and occupied only 13% of the total area. Four vc types were isolated from the xylem of the Alton wych elm, of which one was isolated from five points around the circumference of the trunk and the others from one point each. These four pathogenic phase vc types were tested against representatives of the AL-E bark vc types. Two of the bark vc types were shown to be compatible with two from the xylem (see Figure 3.6), one in particular occuring in several discontinuous areas around the edges of the gallery systems on which the grid was centred.

3.5 DISCUSSION

3.5.1 Comparison of the Alternative Isolation Methods

Despite the significantly lower proportion of *S.multistriatus* galleries from which *O.ulmi* was isolated using the dilution method, there is no real suggestion of major discrepancies between the results of the dilution and bark chip isolation methods when other factors are taken into consideration. Even with the limited data for *S.scolytus* the range in the number of vc types per gallery for each beetle species was virtually the same for the two methods, and when comparing results between beetle species there was no trend towards fewer or greater numbers of vc types per gallery for a particular method (Figure 3.2). It can therefore be tentatively concluded that similar results would have been obtained for the establishment of *O.ulmi* in expanding galleries using either method. This is perhaps because *O.ulmi* mycelium actively growing in bark will be producing spores. Consequently, methods favouring isolation from mycelium or spores, as Lea (1977) suggested is the case for the chip and dilution methods respectively, will tend to give similar results.

However, a conceptual distinction must be made between sampling from the spore inoculum deposited by beetles in entrance holes and early maternal galleries, and sampling from mycelia, even if of limited extent, which have established from this inoculum. Clearly, the small surface area of an entrance hole restricts the opportunities for chip sampling, and the detection of a large number of different vc types, such as that which would be present in an ascospore inoculum, would be unlikely. Despite this limitation, isolations from entrance holes did not result in a lower mean number of vc types compared to isolations from the later stages of expanding galleries (Table 3.2). This does not exclude the possibility that some vc types present in the original spore inoculum fail to become established, particularly from a blob of ascospores. Thus, the results must be considered as largely representing the genetic structure of early established mycelia, rather than the structure of the initial spore inoculum from which the mycelia are derived. A detailed investigation of the pre-mycelial stage would probably be best carried out using the dilution method, but would be made difficult by the short period of time between spores being deposited in the gallery, and subsequent spore germination and development of mycelia.

3.5.2 The Establishment of *O.ulmi* in Bark

The similar results for the four expanding gallery stages (Figures 3.3 and 3.4) show that the mosaic pattern is initiated at stage 1 in most galleries. Furthermore, they suggest that once a mycelium has established, vc types are unlikely to be eliminated by competition during the ensuing colonisation period. The larger number of vc types per gallery for *S.scolytus* (Figure 3.5) probably reflects the greater surface area available for carrying spores compared to *S.multistriatus*. Indeed, the larger elm scolytids have been shown to carry more spores (Webber & Brasier, 1984; J.F. Webber, unpublished data), and will have larger pupal chambers which must increase the chances of beetles becoming contaminated by more than one vc type. The smaller number of vc types in *S.multistriatus* galleries will be at least partly compensated by the smaller area of bark occupied by each gallery system, and the consequent higher breeding density. Therefore, the overall pattern of the mosaic, as represented by the means and ranges of the areas occupied by different vc types, is likely to be similar for both species. The combined evidence from various bark samples suggests that this is indeed the case.

The proportion of galleries from which *O.ulmi* was isolated, namely 100% of *S.scolytus* galleries and 75% of *S.multistriatus* galleries, is much higher than the number of beetles carrying *O.ulmi* after flight and the small number of feeding grooves contaminated with *O.ulmi* as reported by Webber & Brasier (1984). However, the local abundance of breeding material at Barrow Hill Farm and consequent short flight distances for newly emerged beetles may have reduced the loss of spores prior to gallery construction. In large trees dying over several years, some emerging beetles may simply have crawled across the bark to find suitable breeding material. It is also possible that breeding galleries are inoculated with spores from both male and female beetles, and that the high nutrient and moisture content of the inner bark provide a much more favourable environment for spore germination than feeding grooves.

In addition, some of the vc types present as early as Stage 2 originated from the pathogenic phase in the xylem (3.5% of vc types in samples BHF-2 and 3), their release probably assisted by the female beetle scoring the xylem when making a gallery. Assessment of the extent of feedback from the pathogenic phase by vc testing is likely to result in its underestimation, since several different vc types are sometimes present in only a small width of xylem, which could result

in the failure to detect some pathogenic phase vc types. The AL-E vc types which had apparently colonised the bark from the pathogenic phase (see Figure 3.7) tended to occupy areas around the edges of a gallery, suggesting that the pathogenic phase genotypes were growing into unoccupied areas of bark between the expanding galleries.

This evidence for feedback from the pathogenic phase at the very first stages of bark colonisation emphasises the importance attributed to it by Webber & Brasier (1984). Far from being locked in the xylem as previously suggested (Fransen, 1939; Gibbs & Brasier, 1980) pathogenic phase isolates are likely to have an important role during the saprotrophic phase. The colonisation of expanding galleries from a mycelium already present in xylem vessels may confer an advantage over vc types introduced as spores by the breeding beetles, in addition to the advantage of increased cytoplasmic fitness resulting from passage through the pathogenic phase (Brasier, 1986a,c). The rate of feedback shown both by Webber & Brasier (1984) and in this study, is likely to be important in maintaining the pathogenic fitness of the population.

3.5.3 Population Structure in Fully Colonised Bark

The spatial mosaic of vc types in fully colonised bark is very similar to the pattern identified by Lea (1977). However, since the method available to Lea restricted the number of consistently identifiable morphological types to six (although variation was also noted within each type), an area apparently occupied by a single morphological type may sometimes have been occupied by more than one vc type. Indeed, the identification of vc types represented by only one or two isolations would have been extremely difficult using morphological criteria, and clearly parts of bark sample BHF-6 (Figure 3.7) with several genotypes in a small area, could only be fully analysed using the vc system. It is a reflection of the diversity in *O.ulmi* that the mosaic pattern was originally determined on the basis of only six morphological types, and that the vegetative compatibility system with its capacity for distinguishing genotypes reveals essentially the same pattern but with greater resolution.

The absence of a clear relationship between the structure of the mosaic and the positions of the beetle galleries in BHF-6 (Figure 3.7), sampled in spring, and the demontration of such a relationship in AL-E (Figure 3.6), sampled in autumn, is probably a result of the mycelial interactions and periods of recolonisation during the

overwintering period in the former. Thus in AL-E the galleries had only recently met and consequently a correlation with the mosaic might be expected. The greater lontitudinal extent of the areas occupied by many vc types in BHF-6 can be accounted for by faster growth along the grain, since phloem vessels are vertically elongated and there are fewer cell walls for the fungus to grow through (Webber et al., 1987). The same feature results in elongated, lens-shaped lesions when isolates are inoculated into healthy elm bark, as noted by Lea (1977) and Webber (1979).

The large number of different vc types identified in BHF-6, a total of 114 in an area of 536 sq cm, apparently exceeds the 78 that would be predicted from the number of *S.scolytus* and *S.multistriatus* galleries present and the mean number of vc types expected per gallery for each species. Possibly, the smaller areas of BHF-6 occupied by large numbers of vc types are regions recolonised from ascospores. Although not every possible paired combination of the 114 vc types was tested, the strategy used to analyse the grids indicates that few if any of the widely separated BHF-6 vc types were compatible, other than the small number of NAN supergroup isolates.

Only a small proportion of the vc types in BHF-6 colonised large areas of the sample (see Figures 3.7 and 3.8), the majority (88%) occuping areas of 5 sq cm or less. On this basis many pupal chambers would be formed within an area occupied by a single vc type, a point which apparently conflicts with the large number of different vc types found in expanding galleries. However, mycelial penetration between different vc types, the subsequent enhanced sporulation, and the fertilisation of protoperithecia by mites, could lead to a very diverse beetle sporeload. The discontinuous areas occupied by some vc types will also increase the spatial diversity, and may be the result of secondary dispersal by mites and the outcome of competitive interactions. It should also be noted that the grid isolations were colonisation of the outer bark where many pupal chambers are formed may be different. Nevertheless, vc types occupying large areas of the inner bark are probably more likely to colonise the outer bark, and consequently more likely to contribute to beetle sporeloads. Pupal chambers formed in the outer bark might also be occupied by a greater diversity of vc types, since maternal and larval galleries in the inner bark probably act as channels for mites, facilitating secondary dispersal. However, Lea (1977) and in Brasier (1984), found a

comparable diversity at four different bark levels including the outer bark. A combination of the above factors could account for the large number of vc types recorded per expanding gallery even if most original sporeloads consisted mainly of conidia of one or two genotypes with occasional ascospores.

4 COMPARISON OF THE STRUCTURE OF SAPROTROPHIC AND PATHOGENIC PHASE POPULATIONS

4.1 INTRODUCTION

The results of the preceding chapter confirm the mosaic pattern of population structure and demonstrate a high level of diversity in saprotrophic phase populations, even in a small area of bark. A complementary approach to the above analysis of population structure is to measure certain gene frequencies. The method devised by Brasier (1984) involves estimating: the frequency of the NAN vc supergroup, a specific and frequent although otherwise little characterised combination of vc genes; the frequency of the vc supergroup *w* allele, a clearly identifiable vc locus; and the frequency of the A mating type. The characteristics and genetic basis of the different vc reaction categories are described in Chapter 1.5. The supergroup *w* allele frequency can be calculated from the frequencies of the different vc reaction categories against the supergroup, since isolates carrying the supergroup *w* allele will give either a compatible, line, line-gap or narrow reaction against the supergroup.

A number of pathogenic phase populations have been examined in this way by Brasier (1984, 1986a). A higher frequency of supergroup isolates and an associated lower A mating type frequency occurs at epidemic fronts as compared to post-epidemic populations, and is thought to reflect the selection pressures associated with the different levels of disease. If there are any differences in the selection pressures acting during the saprotrophic and pathogenic phases then these too might be reflected in differences in population structure.

Although the above approach will allow populations to be compared, it will not provide much information about the genetic heterogeneity of the non-supergroup component of the population (ie those isolates giving wide or narrow reactions against the supergroup). It can be assumed from studies on the genetic control of vegetative compatibility (Brasier, 1984) that all those isolates giving narrow reactions against the supergroup will also give narrow reactions against each other, since they should all have the same *w* allele. They may also however, give line, line-gap or compatible reactions if they are isogenic at further vc. loci, and will of course all give wide reactions against isolates giving wide reactons against the supergroup. The latter isolates should also fall into a number of groups sharing the same *w* allele and giving narrow reactions or less against each other, assuming the

model of a single multi-allelic *w* locus to be correct.

The mosaic of vc types recorded in fully colonised bark shows that the saprotrophic phase population is indeed highly heterogeneous, and implies that most beetles breeding in adjacent areas of bark introduce different *O.ulmi* genotypes. The diversity within the non-supergroup component of a pathogenic phase population has been estimated by Brasier (1984), by vc testng in all combinations 12 isolates from a total sample of 61 collected from around Chichester. Most pairings gave wide reactions, although there were three narrow reactions and a single compatible reaction. More detailed investigation of local saprotrophic and pathogenic phase samples, such as from Mersea Island, would provide a better estimate of the number of *w* alleles and the diversity within the non-supergroup component, and consequently a more detailed picture of the overall patterns of infection and bark colonisation.

4.2 OBJECTIVE

To compare the population structure of the saprotrophic and pathogenic phases in terms of the frequency of the NAN aggressive vc supergroup and its *w* allele, the frequency of the A mating type, and the extent of genetic diversity within the heterogeneous or non-supergroup component of the population.

4.3 MATERIALS AND METHODS

4.3.1 Sampling and Isolation Methods

The saprotrophic phase populations were those derived from the Mersea Island and Alton wych elm samples described in Chapter 3.3.1 and summarised in Table 3.1. The pathogenic phase sampling was also carried out on Mersea Island, in particular at Barrow Hill Farm. Twig samples were taken from diseased trees or saplings with current seasons symptoms and isolations made using the method described in Chapter 2.1.1. The positions of sampled trees were recorded on a 1:25000 scale map of Mersea Island, or a 1:5000 scale plan of the Barrow Hill Farm site drawn from the map.

4.3.2 Analysis of Isolations

i. Frequency of the VC Supergroup:

Isolates were vc tested on ESA, mostly in 4x4 patterns (Chapter 2.3.1) against an NAN vc supergroup isolate to provide data for the frequency of the vc supergroup, and the frequencies of the different vc reaction categories against it. The resulting reactions were identified as either compatible, line-gap, line, narrow or wide. This method also provided mating type data, as described in Chapter 2.4.1. The saprotrophic phase isolates had already been resolved into different vc types during the investigations described in Chapter 3.

ii. Genetic Diversity in the Non-Supergroup Component:

Most of the data for vc reactions between isolates were derived from other vc tests. For example, for the saprotrophic phase isolations from expanding beetle galleries (Chapter 3.3.3) a certain number of vc tests between isolates from different, often adjacent galleries, were carried out incidentally to tests between those from the same gallery. When collated this information gave an estimate of the comparative heterogeneity of isolates brought into the bark by different beetles. The tests of pathogenic phase isolates against a vc supergroup isolate in 4x4 patterns also provided additional information about vc relationships between isolates. The Barrow Hill Farm 1984 pathogenic phase sample was analysed in greater detail, initially by vc testing overlapping groups of 10 isolates in all combinations. Further tests were then carried out to confirm and expand the resulting pattern.

4.4 RESULTS

4.4.1 Frequency of the NAN VC Supergroup

Data for the population structure of saprotrophic and pathogenic phase samples in terms of the frequencies of the NAN vc supergroup, the frequencies of the different vc reaction categories against it, and the A mating type frequency are summarised in Table 4.1. Line-gap and line reactions have been included with compatible reactions since thay can be considered to be functionally equivalent. Different sets of Mersea Island saprotrophic phase data within a season have been pooled. The 1983 pathogenic phase sample was collected from much of the area of Mersea Island. The 1984 and 1985 samples were collected from within the approximately 75 ha area of Barrow Hill Farm, sampling as many fresh infections initiated in the current season as possible. Also shown for comparison are pooled data for two pathogenic phase samples collected in 1983 from predominantly English elm populations in Orsett and the surrounding areas of southwest Essex, one of the original British NAN epidemic areas (C.M. Brasier, unpublished data).

Analysis of the Mersea Island data for any one year showed no significant difference between the saprotrophic and pathogenic phases in terms of the frequency of either the NAN vc supergroup, the supergroup *w* allele, or the A mating type. However, there was a significant change in the frequencies of the three reaction categories against the supergroup between each of the years from 1983-5 ($P<0.001$). There was also a significant increase in the overall frequency of the supergroup *w* allele in the population ($P<0.05$). There was no significant difference in the A mating type frequency.

The clearest difference between the six Mersea Island samples and the pooled 1983 Orsett/southwest Essex sample was the higher frequency of the A mating type ($P<0.05$) in the former (17-29% vs 12%). The Orsett/southwest Essex sample was also significantly different from the 1984 and 1985 Mersea Island samples, although not the 1985 sample, in terms of the proportions of the three vc reaction categories against the supergroup ($P<0.001$). The Mersea Island samples tended to have more narrow reactions against the supergroup but fewer compatible reactions. Consequently there were only small differences in the overall supergroup *w* allele frequencies ($P<0.05$).

4.4.2 Genetic Diversity of the Non-Supergroup Component

Table 4.2 shows the estimated frequency of each vc reaction category

Table 4.1 Population Structure of Saprotrophic and Pathogenic Phase Samples

Sample		Sample size	VC reaction against supergroup* % Fully compatible	% n reaction	% w reaction	% Overall A mating type	% Carrying supergroup w allele+
Alton wych elm Nov 1983		20	20	5 (0)	70 (0)	0	30
Mersea Island							
(Saprotrophic phase (HAY, WF, BHF-1)	July & Nov 1983	101	10 a (0)	8 (13)	82 (29)	25 a	18 a
(Pathogenic phase	Oct & Nov 1983	77	7 a (0)	9 (22)	84 (26)	25 a	16 a
(Saprotrophic phase (BHF-2, 3, 4, 5, TL1)	July & Aug 1984	210	2 b (0)	15 (34)	82 (21)	23 a	17 a
(Pathogenic phase	July & Aug 1984	77	6 b (0)	17 (44)	77 (17)	17 a	23 a
(Saprotrophic phase (BHF-6)	May 1985	114	4 c (0)	32 (7)	64 (22)	29 a	36 b
(Pathogenic phase	Aug 1985	39	5 c (0)	23 (29)	72 (33)	23 a	29 b
Orsett and SW Essex (original epidemic site) Pathogenic phase	July 1983	157	12 a (0)	13 (5)	76 (14)	12 b	24 ab

* Percent A mating type in each reaction category shown in brackets.
\+ Sum of compatible, line and narrow reactions against the supergroup.
++ Includes a single line reaction against the supergroup.
Different letters indicate significant differences between the frequencies in each column.
Lettering in the column for compatible reactions indicates differences in the frequencies of all three reaction categories at $P < 0.001$. $P < 0.05$ for A mating type and w allele frequencies.

Table 4.2 Estimated Frequencies of Vegetative Compatibility Reaction Categories between Isolates from Saprotrophic and Pathogenic Phase Samples

Sample		No. of isolates	No. of pairings made as % of total possible pairings	Estimated proportion of each reaction category in total of possible reactions			
				% compatible*		% narrow+	% wide
Saprotrophic phase							
Waldegraves Farm, WF	Nov 1983	28	33	1		99++	
Barrow Hill Farm, BHF-1	Nov 1983	39	17	3		97++	
BHF-2	July 1984	46	12	5)		16	79
BHF-3	July 1984	38	10	1)	a	15	84
BHF-4	July 1984	49	8	1)		12	87
BHF-5	July 1984	59	3	5)		15	76
TL1a	Aug 1984	42	79 x	8	b	23	69
Pathogenic phase							
Mersea Island	Oct & Nov 1983	77	5	1		99++	
Barrow Hill Farm	July & Aug 1984	77	100 x	< 1	c	17	83

* Excludes compatible reactions between NAN vc supergroup isolates.
\+ Includes narrow reactions between isolates carrying the supergroup w allele.
++ No distinction made between wide and narrow reactions.
x Not all pairings actually made. Results of untested pairings inferred as described in text.
Different letters in compatible reaction frequency column indicate significant differences ($P < 0.001$) in frequencies of all reaction categories, using pooled data for BHF-2, 3, 4 and 5 after preliminary analysis showed no significant differences. Samples where no distinction made between wide and narrow reactions excluded from analysis.

within a given sample, excluding compatible reactions between supergroup isolates. Contingency table and chi squared analysis of samples with data for wide, narrow and compatible reactions showed no significant difference between the Barrow Hill Farm samples BHF-1 to 4, but revealed highly significant differences ($P<0.001$) between the pooled data for the BHF samples, Trap Log 1 sample a (TL1a) and the 1984 pathogenic phase sample.

Further vc tests of the TL1a isolations showed that five vc types, and two in particular, were repeatedly isolated from different galleries. If these repeats were considered as single vc types then the diversity of vc types in the rest of the sample was similar to that found in the other bark samples. Four of these five vc types gave narrow reactions against the supergroup, which explains the higher frequency of narrow reactions, and two were A mating type. Isolates from different galleries which gave compatible reactions against each other were assumed to give an identical reaction against other vc types. Many of the wide and narrow reactions compiled for TL1a in Table 4.2 were inferred in this way. The method of testing the 1984 Barrow Hill Farm pathogenic phase sample effectively paired the isolates in all combinations. This allowed them to be assigned to different *w* allele groups such that tests between isolates from the same group gave either narrow or compatible reactions, and tests between isolates from different groups gave only wide reactions. The isolates within each group were tested in all combinations, but once an isolate had been assigned to a group it was assumed that it would give a wide reaction against any isolate from another group. In practice many tests between groups were made while analysing the sample. On the basis of a single multi-allelic *w* locus the isolates of each group share the same *w* allele.

The 77 isolates fell into six *w* allele groups of differing sizes, all of them except the smallest (*w11* allele) comprising isolates of both mating types (Table 4.3). Only one isolate could not be reliably assigned to a group, since it gave doubtful narrow reactions against isolates from two different groups. Excluding the supergroup, seven pairs of isolates gave compatible reactions with each other.

The isolates of each *w* allele group were randomly distributed throughout the sampling area, and the distances between each of the compatible isolates in the above pairs ranged from 20 to 625 m, with a mean of 190 m. The maximum distance between any two sampled

trees was 925 m.

Table 4.3 Frequency of w Allele Groups in the Barrow Hill Farm 1984 Pathogenic Phase Sample

w allele group*	% of sample	% A mating type in group
NAN supergroup w allele (w1)	23	28
w2 allele	22	6
w6 allele	18	14
w13 allele	17	23
w8 allele	13	10
w11 allele	5	0
uncertain	1	(100)
sample size	77	

* w allele code derived from number of first isolate in group.

4.5 DISCUSSION

4.5.1 Frequency of the NAN VC Supergroup

The significant differences in frequency of the vc supergroup, the narrow and wide reaction categories against it, and the A mating type for the Mersea Island and the Orsett/southwest Essex samples shown in Table 4.1, suggest important differences between the two populations. Data from the Alton wych elm sample were excluded from the statistical analysis because of the small number of vc types involved, and although it is likely that there were also differences between this and the above populations it will not be considered further.

The differences may perhaps be best explained by a combination of local variations in selection pressures and genetic drift. Brasier (1986a,1987) has recognised two types of selection acting on *O.ulmi* populations; routine and episodic selection. Routine selection is that to which a fungal pathogen would normally be locally subjected during its disease cycle, tending to maintain long term stability. Episodic selection is that imposed by sudden major changes in one or more key selection components, as in a major epidemic, and is likely to have a destabilising effect leading to changes in population structure.

The Orsett/southwest Essex sample was taken from an area of relatively high 'second phase' disease levels in regenerated English elm root suckers, with a fairly large host resource and beetle population. The population structure can be regarded as fairly typical of the immediate post-epidemic period, with a reduced supergroup frequency and increased A mating type frequency compared to fresh epidemic front populations (Brasier,1984,1986a). This is thought to result from a reduced fitness advantage of the supergroup and an increase in pressures favouring the products of sexual reproduction. It could also be interpreted as an increase in the influence of routine selection, resulting in greater genetic diversity and acting against the vc supergroup which is somehow more favoured by episodic selection during rapidly advancing epidemics.

On this basis the Mersea Island population structure, with its low supergroup frequency and high A mating type frequency, may be due to an even greater impact of selection pressures favouring the products of sexual reproduction. This suggests that episodic selection may have a lower impact, and routine selection be more established than in the post-epidemic populations on English elm. It is interesting to note that the differences in the overall frequency of the

supergroup *w* allele between the two populations is not as great as that of the supergroup itself, suggesting that in the Mersea Island population a once more dominant supergroup component has become dispersed through recombination with the rest of the gene pool. The supergroup *w* allele was certainly the most frequent *w* allele in the Barrow Hill Farm 1984 pathogenic phase sample.

A possible cause of a lower intensity of episodic selection at the Mersea Island site may be a slower rate of disease progress on the local smooth leaved elm. Unfortunately there is little critical data for the comparative pathogenicity of *O.ulmi* on smooth leaved elm, but its better field performance has been noted. Data from the Forestry Commission's Dutch elm disease surveys from 1971-6 (summarised by Gibbs, 1978a) revealed much lower average infection rates in smooth leaved compared to English elm. The greatest differences were found in East Anglia, with average infection rates there of 0.47 and 1.10 for smooth leaved and English elm respectively. Smooth leaved elm is also widely considered to be much more genetically diverse than English elm (Heybroek, 1976; Melville, 1978; Richens, 1983).

The pattern of xylem streaking in some of the large smooth leaved elm from which the Mersea Island bark samples were taken showed that they had already been heavily infected up to eight years previously. Mr. J. Marriage of Barrow Hill Farm also commented on the number of years taken for large trees to die. This apparent relatively high level of resistance may have a genetic basis. It may also be due to the physiological impact of slow growth. Annual increment measurements taken from three moderately sized trees at Barrow Hill Farm revealed a considerably slower growth rate than three English elm from Alice Holt Forest, Friston Forest and Eastbourne, with means of 2.6 (Barrow Hill Farm) and 5.4 mm radial increase per year respectively. Slower growing trees may have a larger number of shorter, small diameter xylem vessels, whereas long, large diameter springwood vessels are thought to result in susceptibility to *O.ulmi*. Slower growth may also be associated with reduced transpiration rates, and since drought stressed trees, which can be assumed to have reduced transpiration rates, are known to be more resistant to Dutch elm disease (Gibbs & Greig, 1977) slower growing trees may similarly be more resistant.

Some evidence for slow transpiration rates in Mersea Island elms was obtained when an attempt was made to introduce a large

inoculum of *O.ulmi* into the xylem at the base of two trees at Barrow Hill Farm in June-July 1985. Uptake was very limited, and so too were the resulting disease symptoms and the extent and distribution of observed xylem streaking when the trees were felled. Strong streaking was also found in the current annual ring of one tree which did not result from the inoculation, yet this tree showed no external disease symptoms, again suggesting a relatively high level of resistance. If such xylem streaking tends to become buried beneath one or more years growth following the initial infection and the bark remains healthy, then when the bark does become suitable for beetle breeding the extent of feedback from the pathogenic to the saprotrophic phase may be reduced. This might in turn favour more heterogeneous products of sexual reproduction rather than the vc supergroup. The population structure data for the Mersea Island samples did not show differences between the saprotrophic and pathogenic phases in any one year (Table 4.1). This implies that the population structure of both the pathogenic and autumn saprotrophic phases is determined by the structure of the saprotrophic phase at the time of beetle emergence in early summer. However, the significant differences in the frequencies of the vc reaction categories against the supergroup, and to a lesser extent in the frequency of the supergroup *w* allele, from 1983-85 show that changes are taking place in the population, and suggests that the changes occur during the overwintering saprotrophic phase. This is illustrated by the data for the 1984 and 1985 samples. There were no differences between the saprotrophic and pathogenic phase samples in either 1984 or 1985, suggesting that they were determined primarily by the inoculum carried by the beetles. However, the spring 1985 bark sample (BHF-6) was significantly different from the combined summer 1984 bark samples, even though they were both initiated by the same beetle generation. The differences between the two samples were presumably the result of interactions taking place between pupal chamber production in summer 1984, the period from the initial establishment of the mosaic in autumn 1984, and the sampling of BHF-6 in the following spring. This provides additional evidence for considerable dynamic changes taking place during the bark phase. Futhermore, it suggests that in the Mersea Island population selection pressures acting during the pathogenic phase have less effect on the population structure than those acting during the saprotrophic

phase. The data from three years sampling are insufficient to comment on whether the changes in population structure are simply random fluctuations due to different seasonal patterns, genetic drift, or a genuine trend, although the former would seem most likely.

4.5.2 Genetic Diversity of the Non-Supergroup Component

The frequency of wide, narrow and compatible reactions within the non-supergroup component of the various samples shown in Table 4.2 further illustrates the genetic diversity within the population. Compatible reactions between isolates from different galleries (excluding those between supergroup isolates) were generally scarce, even within the few hundred sq cm of each bark sample. Therefore, an isolate establishing in bark is most unlikely to encounter another isolate of the same vc type and is effectively unique, even though on a wider scale isolates of the same vc type may exist in another area of bark.

However, saprotrophic phase isolates are probably derived partly from a local source of beetles sometimes carrying the same vc types, resulting in a higher frequency of compatible reactions between saprotrophic phase isolates than between pathogenic phase isolates. The TL1a sample may have been from an especially local population, attracting beetles from a very small area and resulting in the over representation of a few particular vc types. The Mersea Island pathogenic phase samples were of course taken from a much wider area than any of the saprotrophic phase samples, and would have selected only for those isolates out of the many present in feeding grooves throughout the sampling area which had caused an infection.

The seven pairs of compatible isolates in the Barrow Hill Farm 1984 pathogenic phase sample may be seen as derived either asexually and therefore isogenic, or as identical vc types resulting from random recombination of a finite number of vc loci and alleles, and with different genetic backgrounds. It is possible that two beetles emerging from adjacent pupal chambers could have carried spores of the same genotype, dispersed to feed and then both initiated infections. However, the distances between the pairs of compatible isolates do not suggest that the pairs tend to be close to one another, as might be expected if this is the case, although the whole sampling area is well within the flying capabilities of a beetle. Without further investigation it is not possible to distinguish between the alternatives of asexual dispersal and repeat random combinations of vc loci.

Regardless of these difficulties, the very high level of heterogeneity for vc type strongly implies a regularly outcrossing population. Therefore, although vc strictly separates isolates on the basis of differences at the vc loci only, isolates of different vc type can reasonably be assumed to differ at many other loci. This also implies that the greater the number of vc loci at which two isolates are isogenic, the greater may be the similarities in the rest of the genome.

The frequency of narrow reactions among the Barrow Hill Farm saprotrophic phase vc types (Table 4.2) is in good agreement with the frequency of 15.5% that would be predicted from a total of six *w* alleles at various frequencies, as identified in the 1984 Barrow Hill Farm pathogenic phase sample. Although it is not possible to show that these are indeed multiple alleles at a single locus without extensive genetic investigation, it provides an interesting measure of the number of other vc loci needed to give the 69 different vc types in the sample (counting the supergroup isolates and seven compatible pairs as eight vc types). In addition to six alleles at the *w* locus four other loci, excluding those controlling line and line-gap vc reactions, would give a total of 96 different vc types, which is also close to the estimated 114 vc types in the BHF-6 bark sample. This is in reasonable agreement with the minimum of four loci estimated from a single backcross series by Brasier (1984), since the variation within a population would be expected to exceed that for any two vc types, and is within the range reported for other fungi (Chapter 1.4).

If there are in fact multiple biallelic *w* loci, then rather than six alleles of a single locus it is necessary to postulate three biallelic loci. To allow a narrow reaction to be expressed two isolates would need to be isogenic at all the *w* loci. This would predict an 11.6% frequency of narrow reactions in random pairings of isolates, assuming that there are four other biallelic loci in addition to the *w* loci. This model would give a total of 128 vc types. The model of a single multiallelic *w* locus gives a slightly better fit with the observed frequencies of wide, narrow and compatible reactions in the Mersea Island and other wild populations.

In general, the data presented for the diversity of vc type and the low number of compatible reactions among the non-supergroup component of the population, suggest that this degree of genetic diversity at the vc loci is sufficient to reduce to a very low level the

chances of an isolate encountering another of the same vc type. Consequently, with the exception of supergroup isolates, vegetative compatibility effectively defines an isolate sampled from the pathogenic or saprotrophic phases as a genetically unique individual.

5 INVESTIGATION OF THE PENETRATION EFFECT BETWEEN NAN AGGRESSIVE ISOLATES IN BARK

5.1 INTRODUCTION

It is clear from the preceding chapter that the dynamics of the bark phase play an important part in determining population structure. Since the penetration effect (see Chapter 1.5) is a conspicuous feature of most wide and narrow vc reactions *in vitro* (Brasier, 1984) it is likely that it has a major role in mycelial interactions during the saprotrophic phase. The penetration effect is manifested between isolates opposed on ESA by the production of perithecia and/or synnemata progressively further away from the barrage as the vc reaction develops. The synnemata formed in one isolate have been shown to be produced by the other, opposing isolate (Brasier, 1984). Two aspects of the penetration effect are therefore likely to be important during the saprotrophic phase; firstly that of territorial invasion, and secondly that of a stimulus for the production of both sexual and asexual spores.

It has been implied that the ability to produce synnemata and perithecia is an important determinant of success in bark; for dispersal, providing an overwintering resting inoculum, and eventually contributing to the sporeloads of the new beetle generation. Therefore, any factor adding to the ability of a genotype to sporulate is likely to increase its overall success, which in view of the heirarchical nature of penetrating ability *in vitro* (Brasier, 1984) emphasises its potential importance. Strong penetrators might produce more synnemata as a result of penetrating weaker isolates, and as a form of territorial invasion this may also allow occupation of a larger area of bark.

Although the penetration effect has been investigated in some detail *in vitro*, it has not been shown to occur *in vivo*. The following work was intended primarily to determine whether or not penetration does occur *in vivo*.

Penetration cannot be observed directly in bark because synnemata are not usually produced unless the bark is removed and incubated in a damp chamber, and even then they may be formed only erratically. It was therefore necessary to find an alternative method to measure penetration which allowed selective re-isolation from the lesions formed by opposing isolates. The successful use of carbendazim tolerance as a nuclear gene marker (Brasier, 1984) suggested that a

suitable method could be devised involving the use of carbendazim tolerance in conjunction with tolerance to another fungicide. Tolerance to the dicarboximide fungicide iprodione was investigated (see Chapter 2.7 and Mitchell, 1987) and found to give suitable markers.

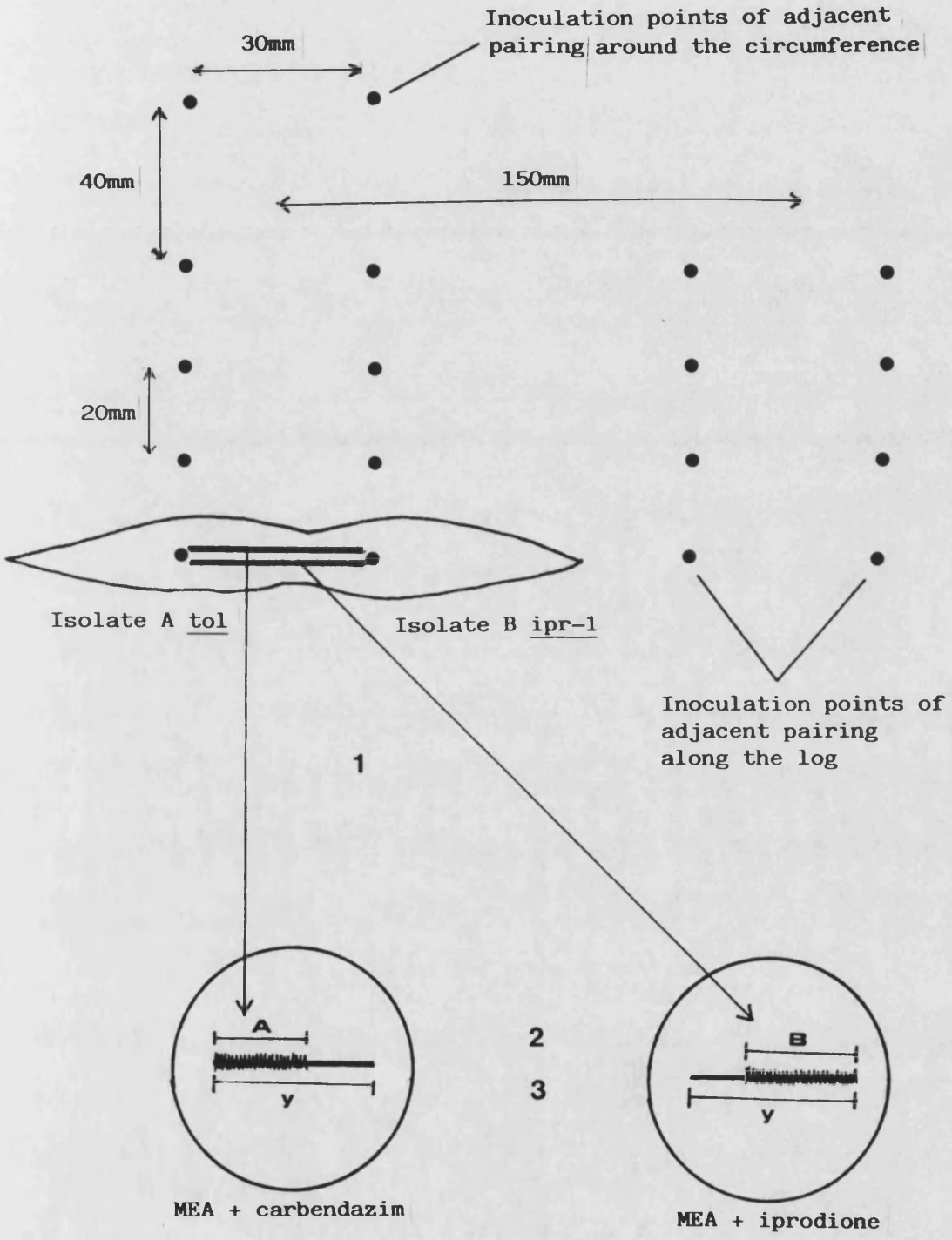
5.2 MATERIALS AND METHODS

Pairs of NAN aggressive isolates giving the following vc reaction categories when opposed on ESA were chosen for the investigation: one control pairing of the same isolate, C112 (vc compatible), one pairing of two supergroup isolates, AL-D18 and H363 (vc compatible), two pairings of B mating type isolates giving wide reactions, HAY-16 and HAY-C14, and AL-D18 and HAY-C14 (fully vc incompatible), and one pairing of isolates of opposite mating type also giving a wide reaction, C112 and S144. For each isolate the methods described in Chapters 2.6 and 2.7 were used to select firstly a mutant tolerant to carbendazim, but sensitive to iprodione, and secondly a mutant tolerant to iprodione, but sensitive to carbendazim.

The selected pairs of isolates were initially opposed *in vitro*, to examine the effects of fungicide tolerance on penetration, and to provide background information on the penetrating ability of the isolates in these particular pairings. Two replicate pairings of each reciprocal combination of fungicide tolerant markers (as described in Section 2.8), and two replicates of fungicide sensitive wild-types as controls, were set up on ESA for each isolate combination. Assessments were made after 26 days incubation at 20°C in darkness. Penetration in the control pairings of sensitive isolates was assessed visually from perithecial and/or synnematal formation and the agar strip re-isolation method (Section 2.8) used for assessing pairings of fungicide tolerant isolates. For the investigation of penetration *in vivo* modifications were made to the selective re-isolation method used *in vitro*. Six week old logs of a healthy English elm from Friston Forest, East Sussex were inoculated using the basic method described in Section 2.9. Inoculations were made in bands 150 mm apart, with four inoculations of one isolate, 20 mm apart, facing four isolations of the second isolate 30 mm away along the grain (Figure 5.1). A gap of 40 mm was left between each set of eight inoculations. It was thought necessary to make four inoculations of each isolate to increase the chances of the lesions meeting, since they follow the grain closely and its direction cannot be determined without removing the bark. English elm has a particularly twisted grain pattern.

Reciprocal pairings in elm bark of the fungicide tolerant markers were made for each combination, except for the pairing of the same isolate, where instead the number of replicates was doubled. Four or five replicates of each reciprocal combination were inoculated. The

Figure 5.1 Measurement of Penetration in Elm Bark using Nuclear Markers



1 Strips of bark from between inoculation points plated out on fungicide-amended agars

2 Mycelial growth of tolerant isolates measured after incubation

3 Combined penetration calculated as (A + B) - y

● Inoculation point

logs were incubated at 17-18°C for 13-14 weeks, and rotated a quarter turn each week.

Assessments were carried out by re-isolation, after carefully removing the bark surrounding each set of inoculations using a mallet and chisel. The exposed surface of the inner bark was cut away and adjacent narrow strips (c. 1-2 mm wide) taken along the length of suitably meeting lesions between the two inoculation points (Figure 5.1). One strip was plated out on MEA + 0.5 ppm carbendazim and the other on MEA + 5.0 ppm iprodione, noting the orientation of the strips to each inoculation point. Growth of *O.ulmi* from the strips onto the selective media was measured after 2-3 days incubation at 25-27 °C in darkness. This high incubation temperature was used since the experiment descibed here was part of a larger experiment investigating the interaction between isolates of the aggressive and non-aggressive subgroups opposed in elm bark. The length of each strip was recorded as a measure of the distance between the inoculation points.

The method provided a reasonably accurate estimate of the total length of the lesion produced by each isolate. However, it was not possible to discern the point at which the lesions had first met (equivalent to the junction between the colonies of isolates opposed *in vitro*), since the method only allowed measurement of the overlap between the two colonies. This overlap was considered to represent the sum of penetration into each isolate, and will be referred to as combined penetration between the two opposed isolates.

The extent of penetration between two opposed isolates was calculated as:

(Length of lesion A + Length of lesion B) - Distance between inoculation points, Y (see Figure 5.1).

Wider, thicker strips from some of the pairings were incubated on water agar containing cycloheximide, streptomycin, carbendazim and iprodione at the usual concentrations to prevent growth on the agar. Stab isolations were made from synnemata developing after 5-7 days incubation at 25-27 °C in darkness, and their positions recorded. Any *O.ulmi* growing from the stab isolations was tested for tolerance to carbendazim and iprodione by assessing growth on MEA containing each fungicide after 1-2 days incubation.

5.3 RESULTS

Penetration data for pairings *in vitro* are shown in Table 5.1. The values for the extent of combined penetration in compatible and wide (fully incompatible) reactions respectively were significantly different ($P<0.001$). The fungicide tolerant mutations themselves did not have any effect on penetration, that is, there was no overall difference in the penetration of iprodione tolerant isolates by carbendazim tolerant isolates and *vice versa* (Table 5.2). Also shown for comparison in Table 5.2 are data for penetration between fungicide sensitive isolates, assessed from the presence of synnemata. The extent of lesion development in bark was poor. Even when the lesions grew well the twisted grain pattern and the way in which lesions closely followed the grain meant that many of the opposing lesions grew past each other, despite the short distance between the inoculation points. These two factors reduced the number of assessable replicates. Two opposed lesions are shown in Plate 2.4.

Iprodione tolerant isolates tended to produce smaller lesions than carbendazim tolerant isolates. In a parallel experiment, using some of the *in vivo* pairings as controls (Chapter 9), this difference was shown to be highly significant. In some replicates the small lesions produced by iprodione tolerant isolates meant that opposed lesions failed to meet even when aligned along the grain.

Mean values for combined penetration between isolates opposed in bark are shown in Table 5.1. Despite considerable variation between replicates analysis of variance showed that combined penetration *in vivo* was again significantly greater ($P<0.001$) in wide (fully incompatible) vc reactions than compatible reactions. Much of the variation around each mean lay in the number of replicates in which there was no overlap of the opposing lesions, due partly to the problems described above. Contingency table analysis showed that there were significantly more pairings without an overlap in compatible reactions than in wide reactions ($P<0.05$).

The re-isolations from synnemata following damp chamber incubation gave insufficient data to allow comparison with results from the strip isolations. This was due mainly to the low numbers of synnemata produced by the iprodione tolerant isolates.

Table 5.1 Penetration between NAN Aggressive Fungicide Tolerant Isolates Opposed in Elm Bark and on ESA

Opposed isolates	vc reaction category	Mean combined penetration (mm)			
		Sensitive isolates	Fungicide tolerant isolates		
		On ESA*	On ESA+		In elm bark ++
C112 v C112	compatible	0	8.5 ± 2.1	a	1.4 ± 1.4
A1-D18 v H363	compatible	0	6.0 ± 1.4	a	0.8 ± 1.4
HAY-16 v HAY-C14	wide (incompatible)	24.5 ± 0.7	38.3 ± 3.5	b	7.6 ± 5.8
AL-D18 v HAY-C14	wide (incompatible)	11.0 ± 1.4	29.8 ± 4.6	b	6.6 ± 5.8
C112 v S144	wide (incompatible) (opposite mating type)	16.5 ± 3.5	27.3 ± 9.2	b	11.7 ± 3.0

* Means of 2 replicates assessed from the presence of synnemata.

\+ Means of 4 replicates (pooled data for reciprocal pairings of fungicide tolerant markers).

++ Means of 8 replicates (pooled data for reciprocal pairings of fungicide tolerant markers) except for HAY-16 v HAY-C14 which is a mean of 7 replicates.

Means followed by a different letter are significantly different ($P < 0.001$).

Table 5.2 Comparison of Penetration on ESA for Reciprocal Pairings of Vegetatively Incompatible Fungicide Tolerant Isolates

Opposed isolates	Mean penetration (mm)*					
	Fungicide sensitive isolates+		Marker combination			
			Carbendazim v Iprodione		Iprodione v Carbendazim	
HAY-16 v HAY-C14	6.5++	18.0	21.0	14.5	20.0	21.0
AL-D18 v HAY-C14	5.0	6.0	18.5	9.0	17.0	10.0
C112 v S144	9.5	7.0	13.0	8.0	18.0	15.5
Mean	7.0 ± 2.7	10.3 ± 6.0	17.5 ± 4.6	10.5 ± 3.4	18.3 ± 1.8	14.4 ± 5.8

* Means of two replicates, assessed after 26 days incubation at 20°C.

\+ Assessed from presence of synnemata.

++ First figure is penetration of first named isolate by the second isolate, and vice versa for the second figure.

5.4 DISCUSSION

The above results demonstrated that the penetration effect occurs between vegetatively incompatible NAN aggressive isolates opposed in elm bark. Although its function remains largely an open question ideas concerning the ecological role of penetration, developed from experimental work in culture, can now be strengthened and extended to interactions taking place during the saprotrophic phase.

There are two points in the saprotrophic phase where any advantages from increased sporulation associated with the penetration effect are likely to be especially important. Firstly, during the initial stages of colonisation from maternal galleries. The strategy employed by individual colonies of *O.ulmi* may involve rapid linear growth in a race to occupy as much bark as possible before encountering other vc types growing outwards from adjacent galleries (Brasier, 1986a). Contact with other vc types may then provide a stimulus to produce synnemata and perhaps perithecia, utilising the captured bark resource, and providing an inoculum for secondary dispersal to other uncolonised areas of bark. Secondly, a similar strategy might be involved in the colonisation of pupal chambers. Where more than one vc type is present increased sporulation may be critical in determining their relative contributions to beetle sporeloads, and therefore to their ultimate success. The territorial invasion aspect of penetration is likely to be important throughout the saprotrophic phase whenever mycelia of different vc types come into contact with each other.

Although the penetration effect has only been investigated between *O.ulmi* isolates, interactions between *O.ulmi* and other fungi should also be considered. A large number of different species can be found in elm bark in association with beetle breeding galleries (Webber, 1979; Webber & Gibbs, 1984; Webber & Hedger, 1986; Brayford, 1983), some of which will be competing directly with *O.ulmi*. It is frequently found (eg Webber, 1979; Rayner & Webber, 1984) that other fungi can stimulate *O.ulmi* to produce synnemata in culture, suggesting that interspecific competition may also stimulate sporulation in bark. Some aspects of the penetration effect may be involved, and confer an advantage to strong penetrators in both inter and intraspecific competition.

Limited experimental work concerning the relationship between the area of ESA occupied and the depth of penetration was also carried

out as part of this investigation. Although the data do not merit detailed description, they suggest that isolates occupying smaller areas may be at a disadvantage. Such isolates appeared to have a reduced capacity both to penetrate and to resist penetration by an opposing isolate occupying a larger area. Similar work by Webber (personal communication) supports this. The inference is that growth rate, size of territory and intrinsic penetrating ability all interact to contribute to an isolate's success during the saprotrophic phase.

Evidence indicating that infection from beetle feeding grooves takes place via a mycelial phase developing from the spore inoculum (Webber & Brasier, 1984; Webber, 1987), suggests this as a further part of the disease cycle where the effects of penetration may be important. However, any advantage to a strong penetrator in feeding grooves is less likely to be attributable to increased sporulation, while the territorial invasion aspect may be of greater importance. The results apparently showed some overlap between compatible fungicide tolerant isolates opposed both *in vivo* and *in vitro* (Tables 5.1 and 5.2). This was probably due partly to slight errors in calculating the lesion lengths in *vivo* and difficulties in accurately marking the junction between the colonies *in vitro*, although some intermingling of hyphae and anastomosed cells containing both types of fungicide tolerant nuclei might be expected. The much lower values for penetration *in vitro* assessed visually in control pairings of sensitive isolates compared to penetration measured using the fungicide tolerant nuclear markers, were probably due partly to poor synnematal production resulting from continuous incubation in darkness. The front of penetration is also likely to be ahead of the production of synnemata.

Even though the fungicide tolerant mutations were not shown to have an effect on penetration, there were differences in relative penetrating abilty between marked isolates compared to assessment from synnemata in pairings of sensitive isolates. However, this may not be a fair comparison due to the unfavourable incubation conditions for the production of synnemata, as descibed above. In addition, assessments were made after 26 days, which is rather sooner than would be suitable for measurement of synnematal penetration.

Although it has been demonstrated using nuclear markers (Brasier, unpublished data) that penetration *in vitro* involves mingling of hyphae of vegetatively incompatible isolates, rather than the migration of nuclei of each isolate through the mycelium of the other,

the fate of the intermingling hyphae and the nature of the stimulus resulting in the production of synnemata has yet to be determined. Since penetration between two isolates is frequently bilateral, although rarely equal, it is perhaps not strictly correct to regard it as territorial invasion. Clearly, if the production of synnemata is associated with lysis of the penetrated mycelium, then lysis is also likely to be mutual when penetration is bilateral. However, the greater advantage in terms of teritorial invasion and increased sporulation will be with the stronger penetrator. Alternatively, if contact with an incompatible mycelium is simply a stimulus to penetrate and sporulate, then penetration should be seen as joint occupation of territory, but still with most benefit to the stronger penetrator.

SECTION I - CONCLUSIONS

The mosaic structure of the saprotrophic phase population was confirmed and shown to be initiated by the variety of vc types that become established in expanding beetle galleries, originating both from the spore inoculum carried by the beetles and through feedback from the pathogenic phase. The results did not suggest that once a mycelium has been formed vc types are eliminated by competition at this stage, but suggested instead that some vc types present in the original spore inoculum fail to become established. Although *S.scolytus* galleries were found to have a higher mean number of vc types per expanding gallery than *S.multistriatus* galleries, it is unlikely that this has a significant effect on the mosaic structure. The identification of the mosaic pattern of genotypes in fully colonised bark using vegetative compatibility strongly suggests that the vc system in *O.ulmi* functions to regulate and maintain the mosaic.

The mosaic pattern ensures that most beetles are likely to carry different vc types on emergence, and their dispersal to feed and breed will further maintain this diversity in subsequent pathogenic and saprotrophic phases. The level of diversity was illustrated by the detailed examination of the Barrow Hill Farm 1984 pathogenic phase sample, which also provided an estimate of the number of vc loci and alleles required to account for this diversity - namely, six alleles of the *w* locus, and at least four other loci. It is suggested that the model of a single multiallelic *w* locus epistatic to the other loci fits more closely with the observed frequencies of wide, narrow and compatible reactions than the alternative model of several repeat *w* loci.

The greater diversity in the Mersea Island NAN aggressive population compared to that found in the Orsett/southwest Essex sample could be interpreted as resulting from a reduced impact of episodic selection and increased importance of routine selection. This would then favour the genetically diverse sexually maintained component of the population over the homogeneous asexually maintained supergroup. In accordance with an 'epidemic selection pressure' model (Brasier, 1986a), this might be due to a lower level of susceptibility of the local smooth leaved elm population to the disease, although not necessarily a direct result of host genotype alone, but perhaps also due to interaction with local environmental factors.

Differences in the frequency of the supergroup *w* allele in both the

bark and pathogenic phases from 1983-85 demonstrated considerable changes in the population structure, which may reflect both genotype competition and variations in selection pressure. Demonstration of the penetration effect between fungicide tolerant isolates opposed in elm bark allowed ideas concerning its role in the dynamics of the saprotrophic phase to be strenghtened.

SECTION II VEGETATIVE COMPATIBILITY AND POPULATION STRUCTURE IN THE NON-AGGRESSIVE SUBGROUP OF *O.ULMI*

In order to understand the ecology of the interaction between the aggressive and non-aggressive subgroups, and to establish the causes of the replacement of the latter, both the vegetative compatibilty system and the population structure of the non-aggressive needed to be investigated. The rapid decline of the non-aggressive during the current epidemics has also made such an investigation a matter of some urgency.

The following study was therefore undertaken. It can be divided into two parts, the first examining the vegetative compatibility system, characterizing different vc reaction types and investigating the genetic control of vegetative compatibility; and the second using vegetative compatibility to investigate the structure of saprotrophic and pathogenic phase populations.

6 VEGETATIVE COMPATIBILITY IN THE NON-AGGRESSIVE SUBGROUP

6.1 INTRODUCTION

An ability to distinguish compatible and incompatible vc reactions is prerequiste to investigating population structure using the vegetative compatibility system to identify genetically different individuals. Although a vegetative compatibility system has been demonstrated in the non-aggressive subgroup by Brasier (1984), the different reaction categories have not been as thoroughly described as for the NAN and EAN races of the aggressive subgroup. Characterization of vc reaction categories might also provide further insight into population structure and the way in which it is maintained, particularly if it is possible to determine the genetic basis of the reaction types.

6.2 MATERIALS AND METHODS

The methods used were mostly as described in Chapter 2. In particular, Chapter 2.3.2 for vc testing non-aggressive isolates, and Chapter 2.5 for obtaining single ascospore progeny for investigation of the genetic control of vc.

6.3 RESULTS

6.3.1 Characterization of VC Reactions

Preliminary work was carried out with a diverse group of 28 stored non-aggressive isolates from northern Europe, provided by C.M. Brasier. In a series of three experiments, a total of 111 pairwise vc tests of these isolates on ESA were compared to control compatible pairings of the same isolate, and to standard NAN aggressive vc reactions. The experiments were duplicated to compare initial incubation at 20 and 30 °C in darkness. The possibility of improving the agar medium was investigated when it became obvious that the clarity of non-aggresive vc reactions varied considerably between different batches of agar. The more important variations were in the numbers of synnemata and perithecia produced and the intensity of the mycelial barrage. Initial incubation at 30°C , the optimum temperature for growth of the non-aggressive, and certain amendments to the medium as described in Appendix 1, were generally found to improve the results. However, the clarity of vc reactions still varied greatly between experiments, and although it was nearly always possible to tell compatible from incompatible reactions, these inconsistencies impeded all aspects of the investigation.

Standard control pairings were used to help in characterizing vc reactions and to estimate their clarity in further experiments, once compatible and incompatible reactions could be recognized with confidence. However, most of the different incompatible reaction categories were first recognised from the large number of pairings, totalling several thousand, made during the investigation of population structure described in Chapter 7. Once tentatively identified, more critical experiments were carried out to examine the reaction categories in greater detail. Population structure was investigated using two main samples, the first comprising over one thousand saprotrophic and pathogenic phase isolates collected from Spain in September 1984 by the author in collaboration with C.M. Brasier and J.F. Webber, and by C.M. Brasier in October 1984; and the second comprising 68 pathogenic phase isolates from North America, kindly provided by several workers. These samples will be described in more detail in Chapter 7.

Several different reaction categories were recognised during the course of the investigation, and their characteristics are summarised in Table 6.1 and illustrated in Figure 6.1 and Plate 6.1. Compatible

Table 6.1 Characteristics of Non-Aggressive Subgroup VC Reaction Categories

Reaction category	Description	Extent of penetration (mm)*	
		From synnemata	From fungicide tolerant markers
compatible	Usually either slight increased aerial mycelium at junction, or colonies merge imperceptibly. Abrupt junction if morphologies different.	None	1-5
line	Thin line of aerial mycelium (c. 1 mm wide), denser than for compatible reaction, although the two categories not always distinct.	None	-
line-gap	Narrow gap (1-1.5 mm) of sparse aerial mycelium between the colonies.	None	1-5
narrow	Dense, narrow slightly raised barrage (c. 2mm), with sporulation directly on it, and usually in a band on either side.	Variable. Probably less than for wide reaction.	7-10
North American narrow	Moderately dense narrow barrage (c. 2mm), usually with sporulation on it. Narrow band (3-5 mm) of sporulation either side, much denser in one isolate. A further discrete, diffuse band of synnemata (c. 10 mm) formed into one isolate only.	Variable, but into one isolate only. Maximum 30 mm.	14-18+ and 5-8

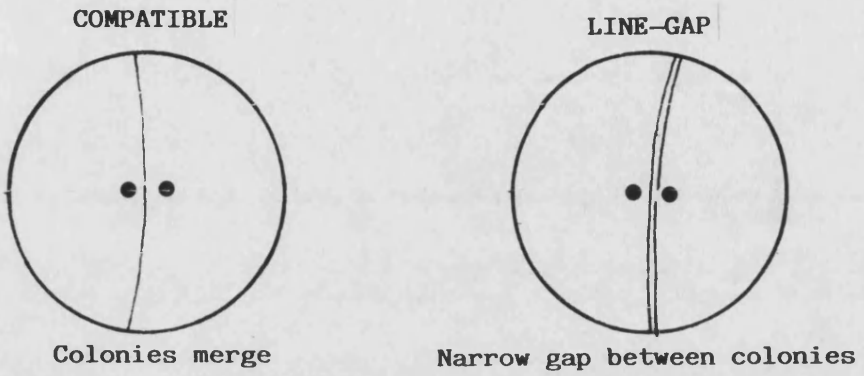
Table 6.1 continued

Reaction category	Description	Extent of penetration (mm)*	
		From synnemata	From fungicide tolerant markers
wide	Usually an area of sparse aerial mycelium (3-5 mm) at junction, with a moderately dense wide barrage (3-5 mm) either side. May appear as a single wider barrage. Sporulation slightly away from each barrage. A discrete, diffuse band of synnemata (c. 10 mm wide) may develop further into each isolate.	Variable. Usually into both isolates. Maximum 30 mm.	10-28

* Measured from synnemata after c. 7 days at 30°C, followed by 3-4 weeks at room temperature in diffuse daylight.
Measured using nuclear markers after about 4 weeks incubation in darkness at 30°C.

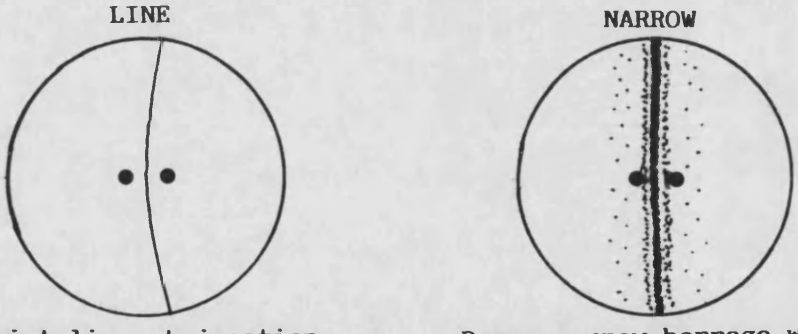
+ Larger measurement into colony in which synnemata were observed.
Smaller measurement into colony without synnemata.

Figure 6.1 Diagrammatic Representation of Non-Aggressive Subgroup VC Reaction Categories



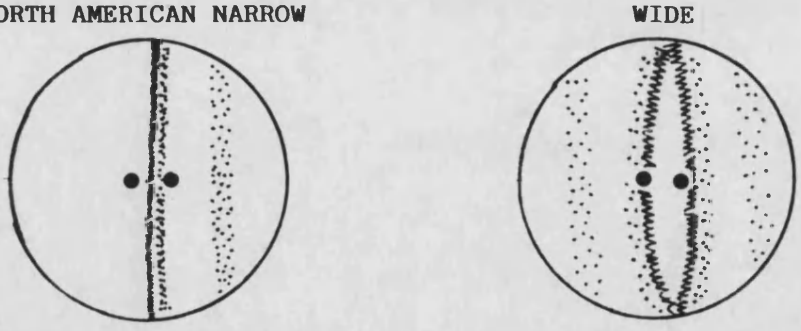
Colonies merge

Narrow gap between colonies



Faint line at junction

Dense narrow barrage with sporulation. Synnemata formed further into each isolate.



Narrow barrage with sporulation. Discrete band of synnemata further into one isolate only

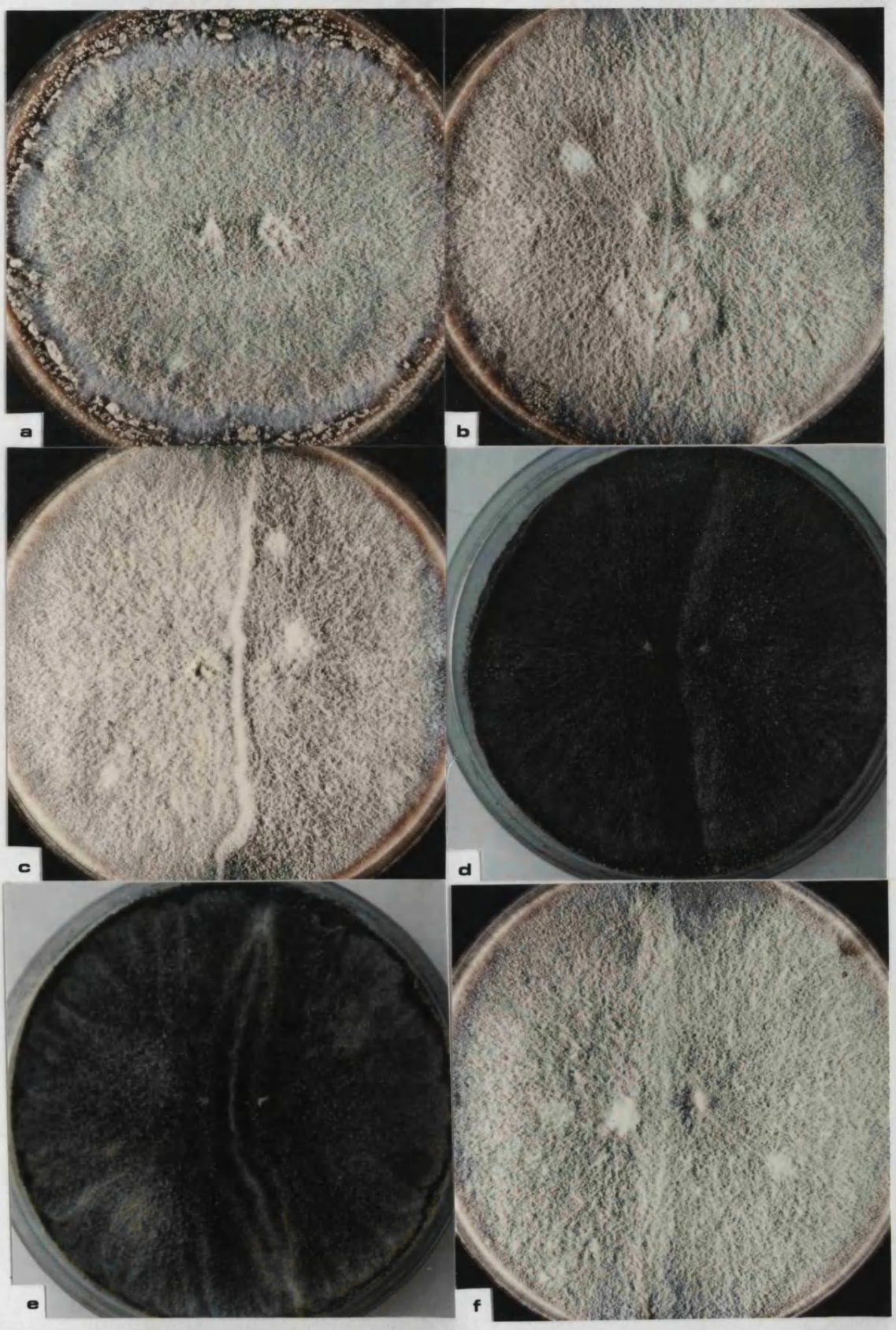
Wider, diffuse double barrages. Sporulation to each side, and a discrete band of synnemata in both isolates

● inoculum

⁘ sporulation

Plate 6.1 Non-Aggressive Subgroup VC Reaction Categories

a Control compatible pairing of the same isolate, b Compatible reaction between different isolates, c Narrow reaction, d North American narrow reaction, e Clear, well defined wide reaction, f Moderately clear wide reaction.



reactions typically had a slightly denser line of aerial mycelium at the junction between the two colonies, although the colonies frequently merged imperceptibly unless there were morphological differences between the two opposed isolates. The most frequent type of incompatible reaction had many features in common with the wide reaction of the aggressive subgroups, such as the formation of a barrage of aerial mycelium between the two colonies and the associated production of synnemata, and was therefore given the same name. A narrow reaction category also showed some similarity with the aggressive subgroup narrow reaction, but it was not always possible to distinguish it from the wide reaction, especially in tests where weak barrages or poor production of synnemata blurred the distinction between them. No definite narrow reactions were identified in the North American sample. In both wide and narrow reactions large numbers of yellow mucilaginous blobs of conidia often developed on the aerial mycelium of the barrage.

Experiments were carried out to test for the occurrence of a penetration effect, and to look for characteristic patterns of penetration in wide and narrow reactions. Synnematal stab isolations from pairings on ESA were vc tested against the respective 'parent' isolates, and it was shown that the synnemata produced within one isolate's colony were produced by the opposing isolate, confirming the penetration effect in the non-aggressive subgroup. Comparison of the depth of synnematal penetration in wide and narrow reactions suggested that it was greater in the former, although considerable variation in the numbers of synnemata produced in each experiment effectively limited the usefulness of this phenomenom for characterizing the two reaction categories. In an attempt to overcome this limitation, the degree of mycelial penetration between iprodione and carbendazim tolerant isolates was measured by selective re-isolation of each fungicide tolerant isolate, rather than from the presence of synnemata. The fungicide tolerant isolates were selected using the methods described in Chapters 2.6 and 2.7, and penetration measured using the comb re-isolation method described in Chapter 2.8.

Although only seven different isolate combinations giving wide or narrow reactions were investigated using this method, the results tended to confirm the differences in synnematal penetration between the two reaction categories (Table 6.1).

The generally low numbers of perithecia produced between

isolates of opposite mating type also limited their use in characterizing vc reactions. Where only a few perithecia were produced they were usually found close to the barrage, although when produced in larger numbers they could be found up to 20 mm on either side of the barrage and were therefore clearly associated with penetration.

A further category of incompatible reaction was seen when the progeny of a cross between two North American isolates, H830 x H827, were vc tested against their parents. Although obviously different from wide and narrow reactions it had some characteristics of each. An unusual pattern of unilateral synnematal penetration was observed such that progeny giving this reaction against H827 were always penetrated, but H827 was not; and progeny giving this reaction against H830 always penetrated H830 but were not penetrated themselves. The extent of unilateral synnematal penetration was similar to that seen in wide reactions (Table 6.1). In several tests using fungicide tolerant markers some mycelial penetration was demonstrated into the isolate in which synnematal penetration was not observed, in addition to the expected penetration of the other isolate (Table 6.1). This reaction category was termed the 'North American' narrow reaction, although it was only observed in the one case and never between wild isolates.

The line-gap (lg) reaction was seen among the North American isolates, although only between two particular vc types, but not among the European isolates. Its characteristics were essentially the same as for NAN aggressive line-gap reactions. Putative line (l) reactions, again essentially as described for the NAN aggressive, were observed at low frequency between wild isolates from the Spanish sample, and also in vc tests against their parents of progeny from a cross of two wild Spanish isolates, SS-A21 x SS-A10 (see below).

All these reaction categories were characterized using a single pairing on a plate, but for the investigation of population structure described in Chapter 7, many tests were carried out in 4x4 patterns (see Chapter 2.3.2). In general, compatible (c), narrow (n) and wide (w) reactions could be readily distinguished on 4x4 patterns on the basis of the following characteristics: c, the merging of colonies; n, the formation of a very narrow slightly raised barrage with synnemata and blobs of mycelial conidia produced directly on it; and w, the formation of a band of synnemata each side of a wider central barrage or sparse area (Plate 6.2).

6.3.2 The Genetic Control of Vegetative Compatibility

The genetic control of vc was investigated by obtaining single ascospore progeny from crosses between parents giving a defined vc reaction category, using in some cases a carbendazim tolerant isolate as one parent to provide a nuclear marker. The progeny were vc tested against both parents, and tested for carbendazim tolerance when appropriate.

In the first series of experiments, using various European isolates, interpretation of vc tests of progeny against their parents was often limited by difficulties in recognising different vc reaction categories and in scoring mating types owing to poor perithecial production. The results for crosses of European isolates presented in Table 6.2 therefore include incomplete mating type data, and in crosses where vc classification was uncertain the frequencies of reaction categories are based on the most reasonable interpretation.

The data were tested for their fit to a series of models to estimate the number of vc loci segregating in each cross. Assuming no linkage, the estimate can be made from the ratio of recombinant vc types among the progeny (those progeny giving an incompatible reaction against both parents) to parental vc types (those progeny giving a compatible reaction against one parent). Clearly, the parental vc types among the progeny will be identifiable to vc genotype, but if epistasis is involved it will not be possible to identify all recombinant vc types to genotype. Therefore, most recombinant vc genotypes must be combined into a single class of recombinant vc phenotypes, although it may be possible to identify recombinant vc genotypes giving lower order vc reactions against one of the parents.

The model for the segregation of a single vc locus predicts that all progeny should be compatible with one parent or the other. The model for the segregation of two unlinked vc loci predicts a 2:1:1 ratio of recombinant vc phenotypes to the two parental vc genotypes. Similarly, for the segregation of three unlinked vc loci a 6:1:1 ratio would be predicted, and so on for additional loci.

The observed ratios of recombinant vc phenotypes to parental vc genotypes for the progeny of European isolates shown in Table 6.2 were compared to those predicted by the models. In all four crosses chi squared tests showed no significant differences from the ratios predicted for the segregation of three unlinked loci. Data for progeny of crosses H8O8 x P82 and H365 x P82 were tested for their fit to a 6:1:1 ratio of recombinant vc phenotypes to parental vc genotypes. For progeny of the cross SS-A21 x SS-A31 two of the recombinant vc

Plate 6.2 VC Test of Non-Aggressive Isolates in a 4x4 Pattern on ESA

Showing compatible (c), narrow (n) and wide (w) vc reactions.

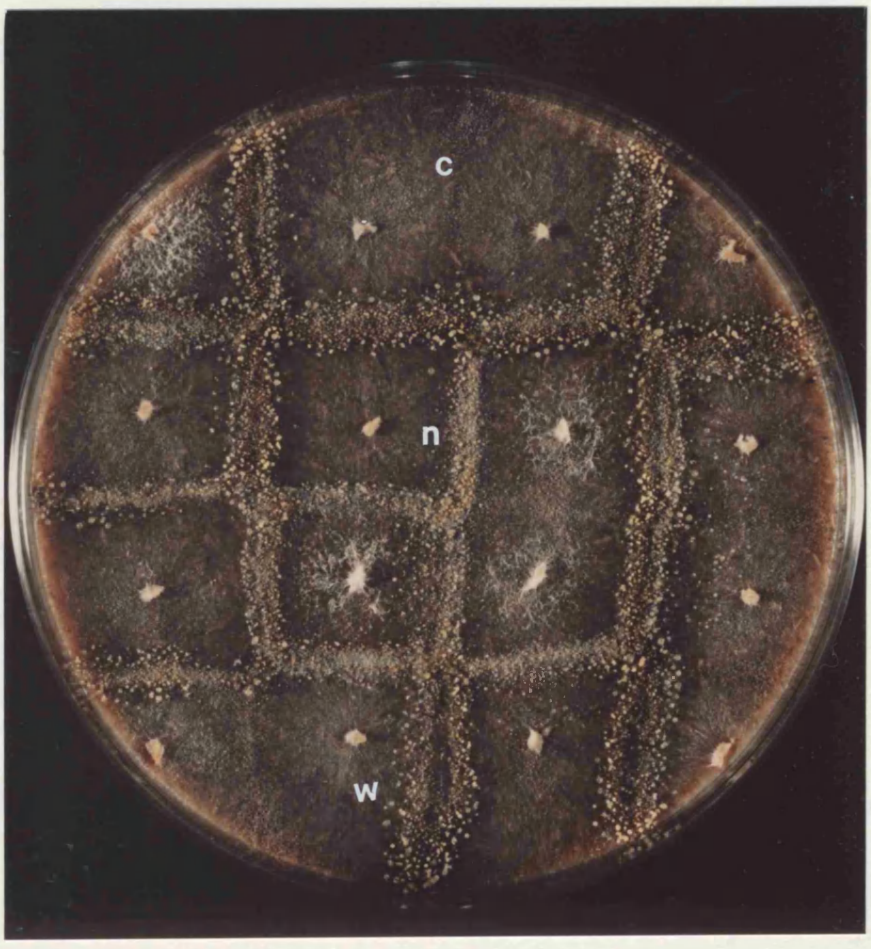


Table 6.2 Segregation of VC Loci in Crosses of European Isolates

Parents* A x B mating type	VC phenotype+		Progeny: Mating type A	B	Unknown	Total in class	Total progeny	X^2 value for ratios of vc phenotypes++
H808 x	w	w	6	11	25	42	58	0.21
P82	c	w	3	0	5	8		
(wide)	w	c	0	6	2	8		
H365 x	w	w	17	49	18	84	113	0.05
P82	c	w	9	0	6	15		
(wide)	w	c	0	13	1	14		
SS-A21 x	w	w	2	1	3	6	36	3.83
SS-A10	n	w	3	1	6	10		
(wide)	w	n	0	0	5	5		
	l	w	2	0	4	6		
	w	l	0	1	0	1		
	c	w	2	0	1	3		
	w	c	0	5	0	5		
SS-A21 x	n	n	9	12	3	24	36	6.11
SS-A31	l	n	0	1	0	1		
(narrow)	n	l	1	3	1	5		
	c	n	2	1	1	4		
	n	c	1	0	1	2		

* All European isolates, except H808 which is from USA. VC reaction between parents shown in brackets.

\+ VC reaction against A mating type parent given first. c, l, n and w = compatible, line, narrow and wide reactions respectively.

++ No significant differences form the ratios expected for the segregation of three vc loci.

genotypes (l/n and n/l) could be separated from the remaining recombinants and the data were therefore tested for their fit to a 4:1:1:1:1 ratio. To adequately explain the data for progeny of the cross SS-A21 x SS-A1O it must be assumed that the w/w vc phenotype of progeny against both parents was mis-scored, and should in fact be either w/n or n/w. When this assumption was made the data were not significantly different from the predicted 4:1:1:1:1 ratio.

A second series of experiments using North American isolates (Table 6.3) was continued until progeny could be confidently assigned to the different reaction categories. Mating type data were complete except for H812 x H815 *tol*. Chi squared analysis showed no significant differences for the independent segregation of vc, mating type and carbendazim tolerance loci. Tests against the models for the number of vc loci segregating in each cross suggested that two vc loci were segregating in the cross of H83O x H827 and one in the crosses of H83O x F1-11 (the latter isolate was an F1 progeny of H83O x H827) and H812 x H815. However, one of the vc loci in the crosses of H83O x H827 and H83O x F1-11 was shown to be linked to the mating type locus, and also to the carbendazim tolerance locus where this was involved.

Table 6.3 Segregation of VC, Mating Type and Carbendazim Tolerance Loci in Crosses of North American Isolates

Parents* A x B mating type	vc phenotype+	carbendazim tolerance	Progeny Amt	Bmt	total	Chi squared values for segregation ratios vc	mt	tol	vc:mt	vc:tol	tol:mt
H830 x H827 (wide)	w n'	-	0	19	87	0.61	0.56	-	88.72***	-	-
	n' w	-	22	0							
	w c	-	0	21							
	c w	-	25	0							
H830 tol x H827 (wide)	w n'	+	0	8	49	4.80	0.18	0.51	56.76***	22.67**	21.32**
		tol	0	0							
	n' w	+	1	0							
		tol	8	0							
	w c	+	0	13							
		tol	0	4							
	c w	+	4	1							
		tol	10	0							
H830 x F1-11 ++ (North American narrow)	w c x	-	21	0	56	2.57	3.50	-	58.14***	-	-
	c w x	-	1	34							
H812 x H815 tol (line-gap)	lg c	+	4		21	1.32	-	0.01	-	7.00	-
		tol	9								
	c lg	+	7								
		tol	1								

* VC reaction between parents shown in brackets.

\+ VC reaction against A mating type parent given first. c, lg, n, n', w = compatible, line-gap, narrow, North American narrow and wide reactions respectively.

++ F1-11; progeny of H830 x H827, giving a wide reaction against H827.

x The F2 classes gave wide and narrow reactions against H827 respectively.

a Chi squared values marked ** and *** differ significantly from the expected ratio at $P < 0.01$ and $P = 0.001$ respectively.

6.4 DISCUSSION

6.4.1 Characterization of VC Reactions

Non-aggressive subgroup vc reactions were not found to differ essentially from those of the aggressive subgroups, although there were obviously differences in detail. It is therefore reasonable to suggest that the physiology, functions and genetic basis of the vc system are broadly the same for all *O.ulmi* subgroups, as would be expected from their ecological similarities and near conspecificity.

The most conspicuous difference between vc reactions in the different subgroups was the much lower intensity of non-aggressive vc reactions in terms of barrage formation and the production of synnemata and perithecia, even at optimal conditions for growth rate and with increased levels of nutrients in the elm sapwood medium. The lower fertility of the non-aggressive subgroup *in vitro* has been previously noted by Brasier (1984,1986a), and suggested as a contributory factor in its failure to compete with the aggressive subgroups, assuming that fewer synnemata and perithecia are also produced by the non-aggressive in bark.

The non-aggressive wide and narrow reaction categories showed similarities to those of the aggressive subgroups in terms of the formation of a mycelial barrage and of synnemata associated with penetration. However, the enhanced production of conidia on the aerial mycelium of the barrage is not a feature of vc reactions in the aggressive subgroups. The apparently greater depth of penetration in wide compared to narrow reactions followed the pattern described for the aggressive subgroups (Brasier,1984), although the unreliability of synnematal and perithecial production prevented detailed comparison.

Non-aggressive line-gap and line reactions were very similar to those of the NAN aggressive. Allowing for inaccuracies in the method used to measure penetration, there was no mycelial penetration in compatible and line-gap reactions (Table 6.1), again as found in NAN aggressive compatible and line-gap reactions (Brasier,1984).

6.4.2 The Genetic Control of VC

The results of vc tests of progeny against parents showed that the vc system in the non-aggressive is under multigenic control (Tables 6.2 and 6.3). However, the difficulties in consistently separating wide and narrow reactions prevented the clear demonstration of control of these reaction types by allelic differences at a *w* locus, as found in the aggressive subgroups. Crosses between the Spanish isolates SS-A1O,

21 and 31 provided the best evidence for a *w* locus, although the absence of narrow reactions in vc tests of progeny against parents for crosses of other European isolates supports the alternative hypothesis that a difference at any one of several vc loci results in a single category of incompatible reaction. It is possible that narrow reactions were present but not recognised in these vc tests. The line-gap and putative line reactions are almost certainly controlled by single loci (see Tables 6.2 and 6.3), as described for the NAN subgroup (Brasier, 1984).

The results of crosses SS-A21 x 1O and SS-A21 x 31 (Table 6.2) suggested that three vc loci were segregating in each case. Assuming that there is a single locus controlling wide reactions, since SS-A21 and 1O gave a wide reaction against each other they should differ at the putative *w* locus, a single locus controlling narrow reactions, and a locus controlling line reactions. However, since SS-A21 and 31 gave a narrow reaction they should be isogenic at the *w* locus, but differ at two loci controlling narrow reactions, in addition to that controlling line reactions. Although the data are limited, this does suggest that there may be several duplicate or repeat loci controlling narrow reactions in the Spanish non-aggressive population, as also suggested for the Barrow Hill Farm 1984 pathogenic phase sample of the NAN aggressive (Chapter 4.4.2).

The demonstration of linkage of the mating type locus to one of the vc loci in the cross of the North American non-aggressive isolates, H83O x H827 (Table 6.3), was unexpected when compared to the situation found in the NAN aggressive. Due to the limited mating type data for the progeny of European non-aggressive crosses (Table 6.2), it is not possible to be certain that in these isolates the mating type locus is not linked to the vc loci, although this would seem likely. Vegetatively compatible pairings of progeny and parents of opposite mating type are much less likely to produce perithecia, because of the limited extent of penetration and reduced opportunities for fertilisation, as demonstrated for NAN isolates (Brasier, 1984). Therefore, vc tests of progeny against parents would be less likely to reveal their mating types. The mating type frequencies for most of the parental vc genotypes shown in Table 6.2 would have been more or less equal if they were of opposite mating type to the parent of the same vc type.

Although investigation of the genetic control of vc in the North American sample concentrated on the single backcross, H83O x

H827, the contrast to crosses of European isolates is notable. Its significance will be discussed in Chapter 7. It is difficult to comment on the unusual lower order incompatible reaction category (North American narrow) seen between progeny of H83O x H827 and their parents, since it was observed only in this case. The curious pattern of unidirectional synnematal penetration emphasises the close relationship between penetration and incompatibility, and further investigation might therefore provide information about the control of penetration.

7 POPULATION STRUCTURE OF THE NON-AGGRESSIVE SUBGROUP

7.1 INTRODUCTION

An understanding of the population structure and the level of genetic variability within populations of the non-aggressive subgroup is prerequisite for interpretation of experiments on competition between the aggressive and non-aggressive subgroups. The population structure of the non-aggressive has so far received little attention, since there has obviously been greater interest in the aggressive races and the epidemics caused by them. More recently, work with the non-aggressive subgroup has increased in importance as it has become apparent that an understanding of changes in *O.ulmi* populations and the factors leading to change may be critical in eventually restoring the balance between pathogen and host.

Investigations by Brasier (1984) suggested a high level of heterogeneity in pathogenic phase populations of the non-aggressive. A continental sample of 11 isolates from Europe and Turkey, and a regional sample of 13 isolates from Sicily, showed only a single repeat vc group in each when vc tested in all pairwise combinations. Most isolates were therefore of a different vc group, and there was no evidence of a supergroup as found in the NAN and EAN aggressive races. Mating type tests of a total of 232 non-aggressive isolates from Europe and southwest Asia showed a near 1:1 ratio of the two mating types, with 48% A types.

The initial objectives for an investigation of population structure would be to expand the available evidence for the level of heterogeneity and mating type frequencies in pathogenic phase populations, and to compare the saprotrophic and pathogenic phases. Further investigation, for example of establishment in expanding vector beetle galleries, will be restricted by increasingly limited opportunities for field work as the non-aggressive subgroup is replaced by the aggressive. However, once the basic population structure has been determined, it should be possible to infer at least some further information from work with the aggressive subgroup, such as that described in Section I.

7.2 MATERIALS AND METHODS

The sampling and isolation methods for saprotrophic and pathogenic phase samples are described in Chapter 2.1. The vc and mating type test methods used to analyse the samples are described in Chapter 2.3.2 and 2.4.2 respectively.

7.3 RESULTS

7.3.1 Geographical Origins of the Samples

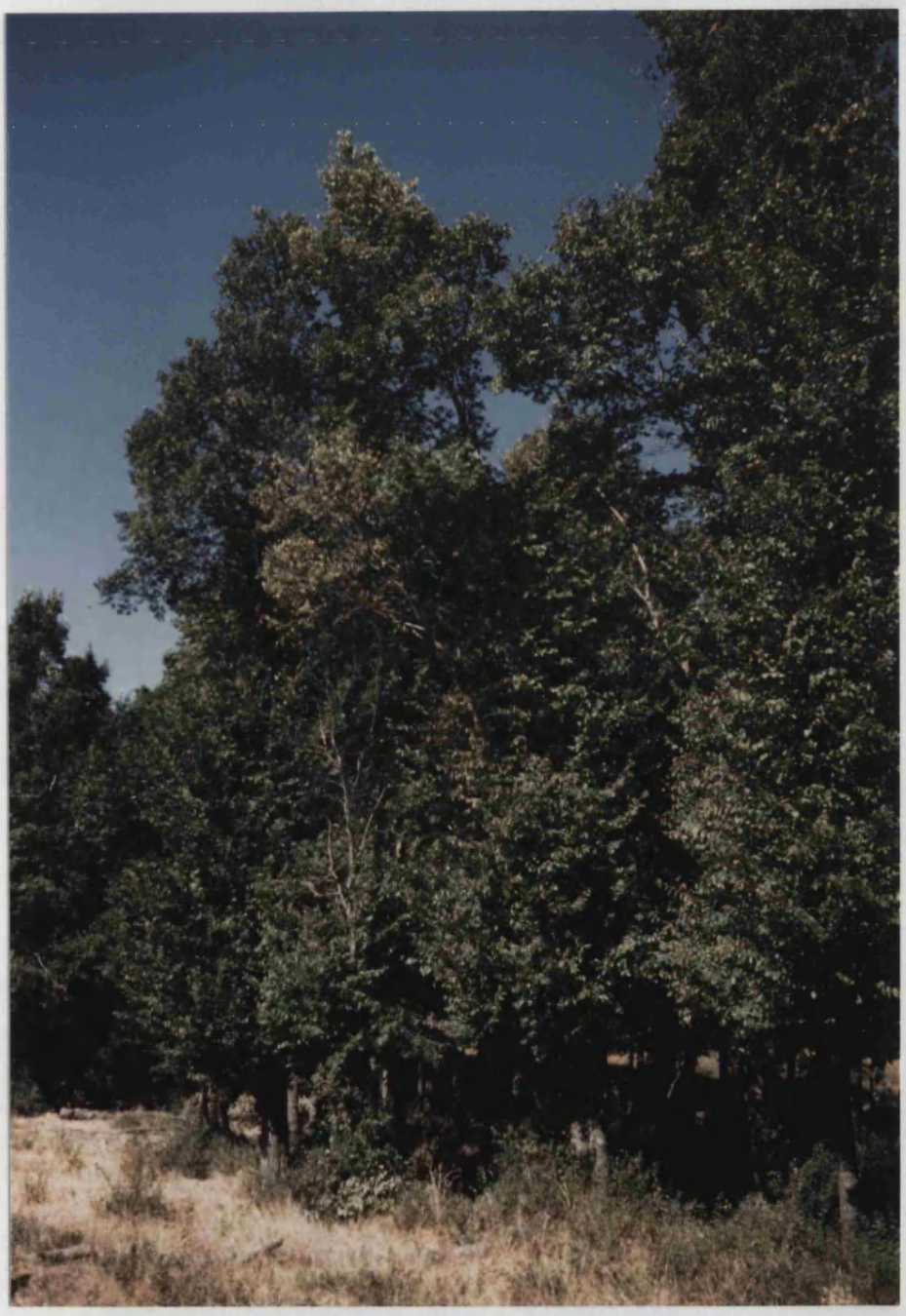
The work was carried out using the Spanish and North American samples mentioned in Chapter 6.3.1. The Dutch elm disease situation in Spain and Portugal was of particular interest because of the advance of devastating epidemics initiated by the NAN aggressive into areas where previously only the non-aggressive subgroup had been present. The elm population of Spain is predominantly *U.carpinifolia*, and elms were seen frequently at field boundaries and in small patches of scrub and woodland, especially alongside watercourses, as well as in town and village streets.

Saprotrophic and pathogenic phase samples were collected from two distinct types of population, defined as endemic or epidemic according to disease status. Endemic populatioms were considered to be directly descended from the earlier epidemics of the 193Os and 4Os (see Chapter 1.1) with only the non-aggressive present. They were characterized by low levels or virtual absence of disease, with occasional local flare-ups when for some reason populations of the vector beetles had been temporarily increased. Elms at these sites were therefore largely healthy and free of disease, although signs of limited crown dieback perhaps attributable to previous flare-ups were often seen. Larger trees killed by the non-aggressive subgroup were rarely or never observed, although death by other causes such as root disturbance, fire or felling was fairly common, and could have initiated local flare-ups in which small trees might have been killed. The site at which the endemic population saprotrophic phase samples were taken is illustrated in Plate 7.1.

Beetle breeding in the trunks and larger branches of dying trees at endemic sites was mostly of *S.multistriatus*, occasionally with some *S.scolytus*. However, breeding galleries of another smaller scolytid, *S.kirschi* Scalitzky, were frequently seen in small branches of stressed but otherwise healthy trees. *S.kirschi* was therefore behaving more as a primary attacker than the other two species. Work by Webber (unpublished data) has suggested that *S.kirschi* is unimportant as a vector of Dutch elm disease, probably because of its small size (2-3 mm, compared to 2.5-3.5 mm and 4-6 mm for *S.multistriatus* and *S.scolytus* respectively), and a smaller proportion of *S.kirschi* adults were found to carry *O.ulmi* spores (7, 44 and 98% for the three species listed as above). The number of spores carried by *S.kirschi* was also

Plate 7.1 Smooth Leaved Elm near Sancti Spiritus, Spain

Non-aggressive infection can be seen on the tree just left of centre.

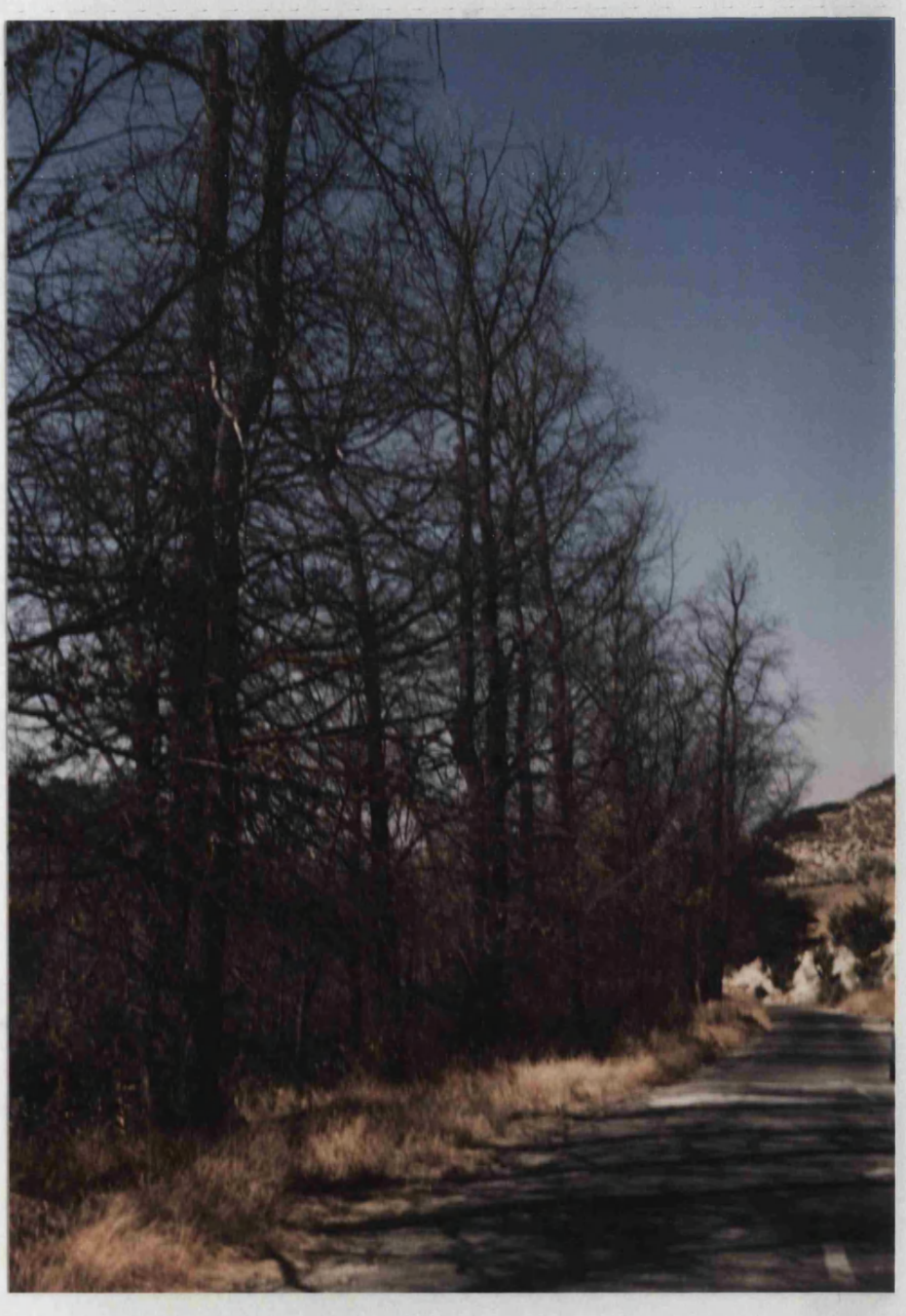


very low. *O.ulmi* was isolated from only 3% of *S.kirschi* breeding galleries (J.F.Webber, unpublished data), compared to 8 and 72% for isolations from *S.multistriatus* and *S.scolytus* galleries at similar sites carried out as part of this investigation.

In complete contrast, sites with epidemic disease levels were characterized by large numbers of dead and rapidly dying elms (Plate 7.2), and presumably enormous populations of vector beetles due to the ready availability of breeding material. It should be noted that the much higher summer temperatures in Spain and Portugal compared to northern Europe would have allowed the beetles to complete several generations in each summer, and may therefore have also increased infection rates. Beetle breeding in the trunks and larger branches of diseased trees at epidemic sites was a mixture of *S.scolytus* and *S.multistriatus*, again with *S.kirschi* in small branches of apparently healthy trees. The endemic population pathogenic phase sample of 26 isolates was collected along a 60 km length of the N620 between Ciudad Rodrigo and Salamanca in Castilla-Leon province, recording the positions of sampled trees on a 1:1000000 scale map. The sample was relatively widespread as a direct of the low disease levels, although healthy elms were frequent at roadsides, field boundaries and in villages. The saprotrophic phase sample was collected from a single site near Sancti Spiritus (Plate 7.1), about 15 km northeast of Ciudad Rodrigo on the N620, from a tree which had been felled from a line of elms along a field boundary ditch and subsequently become colonised by beetles. The general level of disease in the area was moderately low, although there were perhaps two or three hundred elms in the immediate vicinity.

The epidemic population saprotrophic and pathogenic phase samples collected from the small area of the Casa de Campo in Madrid. At this site, a rapidly progressing epidemic initiated by the NAN aggressive was devastating the large elm population of perhaps as many as one thousand trees. Although the epidemic had been initiated by the NAN aggressive this subgroup was present in only 8 out of 62 pathogenic phase isolates (C.M.Brasier, unpublished data). Brasier (1983a) predicted that during the early stages of an epidemic most infections would be caused by the non-aggressive, since the beetle populations, although greatly increased in size, would have been derived directly from those present at endemic disease levels and consequently most beetles would carry non-aggressive spores. Similarly, the

Plate 7.2 Diseased Smooth Leaved Elm at Guadalajara, near Madrid



epidemic non-aggressive population would have been very recently derived from the endemic population which existed prior to the arrival of the NAN aggressive.

The North American pathogenic phase sample of 68 isolates was centred on Vermont in the northeastern USA, with additional isolates from surrounding American states and Canadian provinces, and also from some southeastern and midwestern states. Most of the isolates were kindly provided by D.R.Houston from samples taken in 1980, 1983 and 1986 (Houston, 1985), with others from samples made by J.N.Gibbs (Gibbs *et al.*, 1979), R.J.Iuli in 1984 (Iuli & Campana, 1985), and the collection of E.B.Smalley, mostly sampled in 1970. The state or province of origin, sampling date and source of the isolates are given in Table 7.1.

The isolates were largely from mixed populations of the NAN aggressive and non-aggressive subgroups on the highly susceptible American elm. Consequently, the disease has been at epidemic levels for several decades, and the sample can be considered to represent a truly epidemic non-aggressive population which has been subjected to the selection pressures associated with epidemics, probably since its introduction to North America in the 1930s. Information regarding the location of the isolates was only available as the town nearest to the sampling site.

7.3.2 Population Structure of the Saprotrophic Phase

Most isolations from fully colonised bark sampled from the endemic Sancti Spiritus site in September 1984 were carried out while still in Spain, and consequently only limited time was available for the work. The grid system and bark isolation method described in Chapter 2.1.2 was used for a total of five bark samples all at about the stage of beetle emergence, details of which are given in Table 7.2. A 10 mm grid interval was used for samples SS-A, C and F, and a 20 mm interval for SS-B and D, the latter allowing a greater area to be covered in a given time. Bark from a single diseased elm at the Casa de Campo epidemic site was collected by C.M. Brasier in late October 1984, and isolations made from three samples, CC-1, 2 and 3, using 10 mm interval grid systems (Table 7.2).

The resulting *O.ulmi* isolations were resolved into different vc types in a series of vc tests, mostly in overlapping groups of 16 in 4x4 patterns (Chapter 2.3.1), as decribed for NAN aggressive samples in Chapter 3. Analysis of the grid isolations from fully

Table 7.1. Origins of the North American Isolates

State/Province	Year	No. of isolates	Source
Vermont	1977	1	EBS[a]
	1980	21	DRH[b]
	1983	14	DRH
New Brunswick	1977	1	JNG[c]
Novia Scotia	1977	1	JNG
Quebec	1977	1	JNG
Connecticut	1984	1	RJI[d]
Kansas	1970	4	EBS
Maine	1970	1	EBS
	1977	2	EBS
	1980	3	DRH
	1983	3	DRH
	1986	6	DRH
Massachusetts	1970	1	EBS
New Hampshire	1977	2	JNG
New York	1977	1	JNG
North Carolina	1970	1	EBS
Virginia	1984	3	RJI
West Virginia	1983	1	DRH

[a] From the collection of E.B. Smalley.
[b] Sampled by D.R. Houston (Houston, 1985).
[c] Sampled by J.N. Gibbs (Gibbs et al., 1979).
[d] Sampled by R.J. Iuli (Iuli & Campana, 1985).

Table 7.2. Saprotrophic Phase Samples from Spanish Endemic and Epidemic Sites

Sample	Date	Grid interval (mm)*	Area (sq cm)	Description
Sancti Spiritus (endemic)				
SS-A	23/9/84	10	100	Fully colonised bark with S. multistriatus breeding
SS-B	"	20	260	"
SS-C	"	10	90	"
SS-D	"	20	240	"
SS-F	1/10/84	10	120	"
Casa de Campo (epidemic)				
CC-1	29/10/84	10	252	Fully colonised bark with S. scolytus and S. multistriatus breeding.
CC-2	"	10	159	Expanding S. scolytus and gallery systems, beginning to meet.
CC-3	"	10	108	Expanding S. scolytus and S. multistriatus gallery systems, beginning to meet.

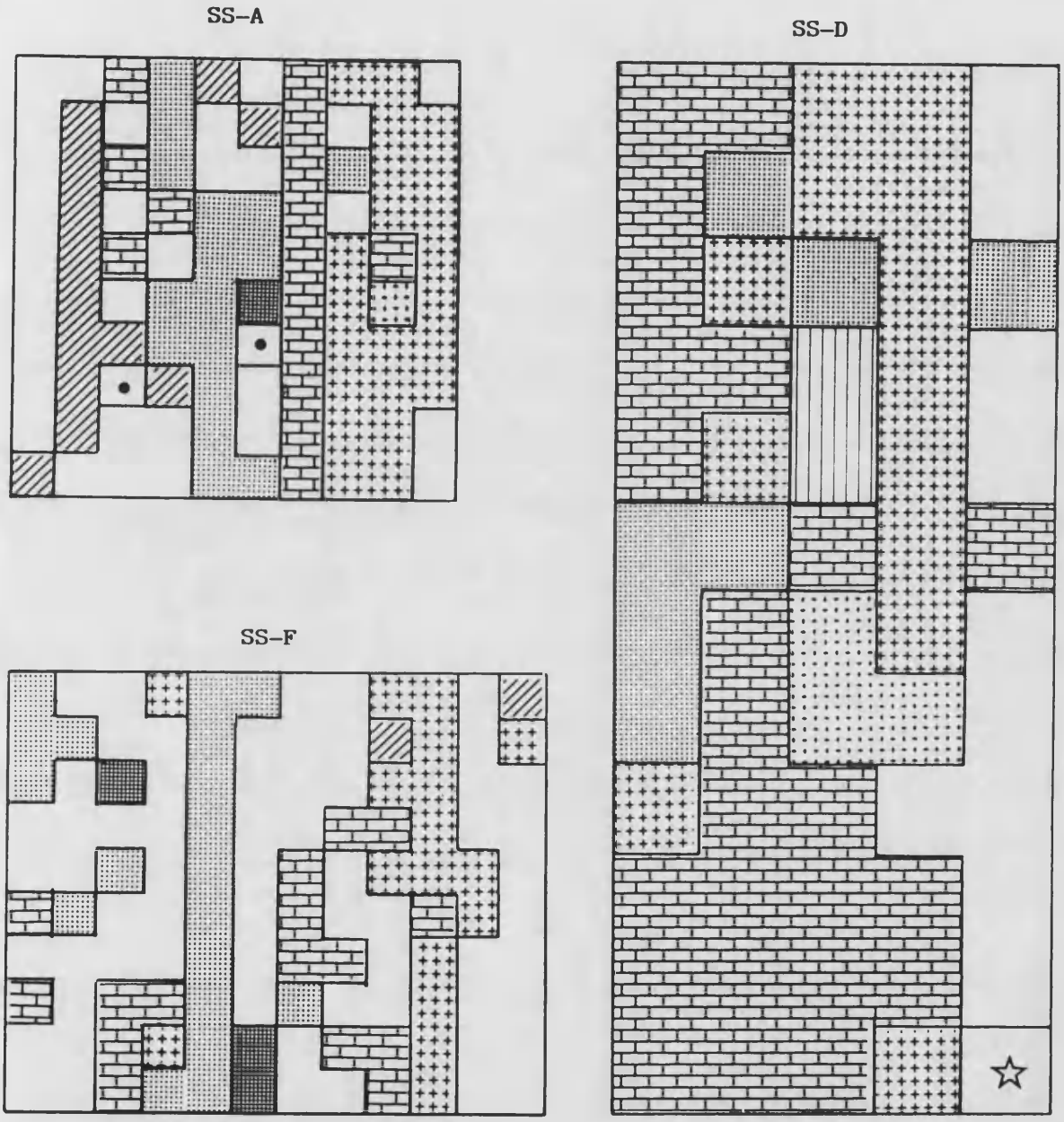
* Isolations made from all samples using grid system and bark chip method (Chapter 2.1.2).

colonised bark from both Sancti Spiritus and Casa de Campo revealed a mosaic of different vc types. Representative samples from each site are shown in Figures 7.1 and 7.2. The relative areas occupied by different vc types in bark samples from the two sites are summarised in Table 7.3, which also includes data for a Mersea Island NAN aggressive sample, BHF-6 (see Figures 3.7 and 3.8). BHF-6 was selected for comparison because of its large size, and also since it had been sampled in May shortly before beetle emergence, a stage more similar to the Spanish samples than the other NAN aggressive sample, AL-E (see Figure 3.6). Although the three representative Sancti Spiritus samples, SS-A, D and F, were taken from the same tree, their precise alignment and the gaps between them were not recorded. Several of the vc types were isolated from more than one of the samples, and so for analysis of the areas occupied by different vc types the three grids have been considered as a single sample.

The relatively small number of vc types present in the Sancti Spiritus and Casa de Campo samples did not allow the data to be presented in terms of frequency distributions, preventing a direct comparison with the NAN aggressive sample BHF-6. However, statistical analysis was made using 2x2 contingency tables, with the two classes comprising vc types approximated as occupying 10 sq cm or less, and greater than 10 sq cm (Table 7.3). The Sancti Spiritus and Casa de Campo samples were compared using exact probabilities because of low expected frequencies, but both were compared to BHF-6 using chi squared tests. No difference was found between the two Spanish non-aggressive subgroup samples, although both were significantly different ($P<0.001$) from the NAN aggressive sample, BHF-6. This suggests that in the two non-aggressive samples vc types occupied roughly similar areas, whereas in BHF-6 a small minority of vc types occupied much larger areas than the rest. Furthermore, this shows that in BHF-6 a larger number of vc types would be expected in a given area. Many vc types in the Sancti Spiritus and Casa de Campo grids occupied discontinuous areas, and the areas were typically elongated along the grain, especially in the former. Similar features were described for BHF-6.

Only *S.multistriatus* breeding was found in the Sancti Spiritus samples, and although maternal galleries could be clearly seen it was not always possible to discern the extent of the associated larval galleries, especially in SS-A. The breeding in CC-1 was nearly all of

Figure 7.1 Mosaic of VC Types in Sancti Spiritus Bark Samples (Endemic Population)

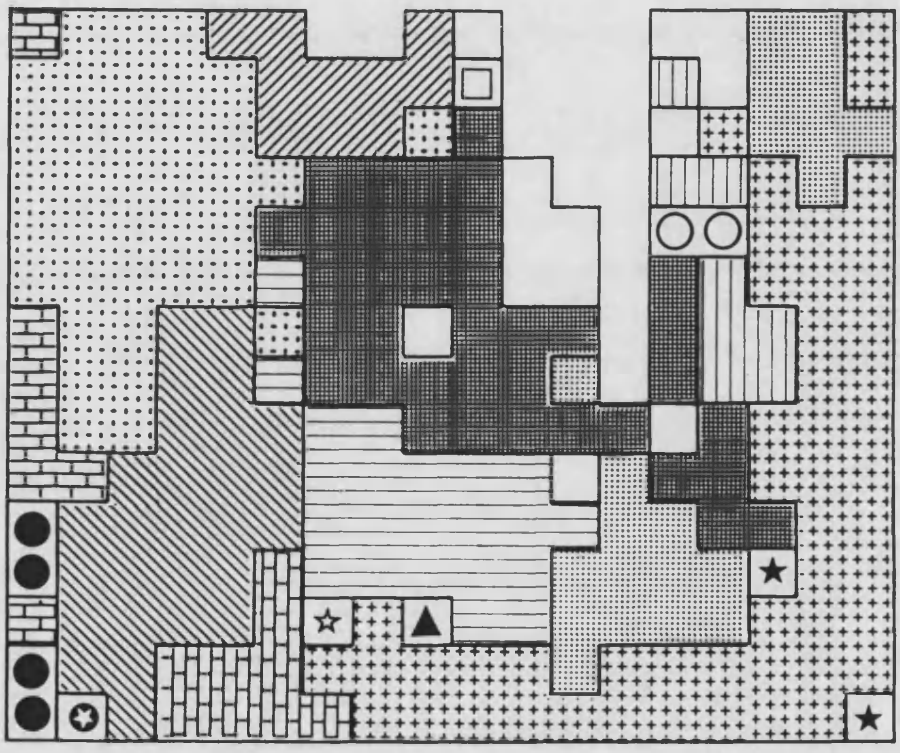


SS-A and F isolated from a 10 mm interval grid, SS-D from a 20 mm interval grid

Each shading pattern or symbol represents a different vc type. No O.ulmi isolated from unmarked areas.

The smallest squares correspond to $1cm^2$.

Figure 7.2 Mosaic of VC Types in Casa de Campo Bark Sample 1 (Epidemic Population)



Isolations made from a 10 mm interval grid. Each isolation has been assumed to occupy an area of $1cm^2$.

Each shading, pattern, symbol or number represents a different VC type. No O.ulmi isolated from unmarked areas.

The smallest squares correspond to $1cm^2$.

Table 7.3. Analysis of Bark Colonisation in Samples from Endemic and Epidemic Non-Aggressive Populations

Sample	Percent successful isolations	No. of vc types	Total occupied area (sq cm)*	Number of vc types occupying areas:+ < 10 sq cm	> 10 sq cm
SS-A, D and F (endemic non-aggressive)	63	9	177	4	5 [a]
CC-1 (epidemic non-aggressive)	96	17	249	11	6 [a]
BHF-G (epidemic NAN aggressive)	90	114	483	104	10 [b]

* SS-D isolations approximated as 1 sq cm per isolation, although isolations were made from a 20 mm interval grid.

+ Different letters indicate significant differences ($P < 0.001$) for the proportion of vc types in each area class.

S.scolytus, but even the maternal galleries were unclear and the extent of larval galleries could not be made out at all. No correlation was found between the areas occupied by different vc types and the gallery systems, although this has not been shown in Figures 7.1 and 7.2. The NAN aggressive was isolated at very low frequencies from the Casa de Campo samples, but not from CC-1.

7.3.3 Comparison of Spanish Endemic and Epidemic Populations

The Spanish saprotrophic and pathogenic phase samples were analysed using a similar strategy to that applied to the NAN aggressive samples (Chapter 4.3.2). Saprotrophic phase isolations were resolved into vc types as described above. Pathogenic phase isolates were vc tested using mostly 4x4 patterns, attempting to test as many of the possible pairings as was considered reasonable for the size of each sample. The tests were designed to include a certain amount of duplication, and pairings giving uncertain reactions were usually retested. All compatible and putative line reactions identified among pathogenic phase isolates from the initial tests in 4x4 patterns were retested as a single pairing per plate. The results are summarised in Table 7.4. Due to the difficulties in distinguishing wide and narrow reactions the frequencies given for these categories should be viewed with caution, although they can be confidently combined as incompatible reactions at least equivalent to narrow reactions.

Most of the isolates in each pathogenic phase sample were of different vc types, with relatively low frequencies of compatible and line reactions. The level of variation in the pathogenic phase samples has been expressed as percent heterogeneity (Brasier, 1988), calculated as the number of isolates divided by the estimated or actual number of vc types x 100%. The putative line reactions have been included as compatible reactions, since two isolates giving a line reaction will differ only at a single vc locus (see Table 6.2), resulting in little functional incompatibility.

In some vc tests reasonably coherent groups of isolates emerged giving narrow reactions against each other and wide reactions against isolates from other groups. However, there were always some conflicting results in each test, and considerable confusion was caused by difficulties in separating wide and narrow reactions in different tests. Many isolates could not be reliably assigned to a particular group and so it is not possible to present any data for the number and frequency of the groups.

Table 7.4. Estimated Frequencies of VC Reaction Categories in Endemic and Epidemic Saprotrophic and Pathogenic Phase Samples

Sample	Sample size*	No. of tests as % of total possible	Estimated frequency (%) of vc reaction categories+				% heterogeneity	% A mating type
			Compatible	Line	Narrow	Wide		
Sancti Spiritus (endemic)								
Saprotrophic phase	15	100	-	-	47	53	-	67
Pathogenic phase	26	99	1	0.5	28	71	81	42
Casa de Campo (epidemic)								
Saprotrophic phase		60	-	-	54	46	-	ND
Pathogenic phase		38	0.5	0.5	32	67	91	45

* Number of vc types for saprotrophic phase samples.
Number of isolates for pathogenic phase samples.

\+ Frequencies of wide and narrow reactions should be viewed with caution due to difficulties in recognising them. Combined frequency of wide + narrow represents total of incompatible reactions at least equivalent to narrow reactions. Line reactions only putative, and may be mis-scored compatible reactions.

++ Data from all Sancti Spiritus bark samples (SS-A, B, C, D and F).

x Data from CC-1 only.

7.3.4 Structure of the North American Epidemic Population

In striking contrast to the heterogeneity of the Spanish samples a very large proportion of the North American isolates was found to be of the same vc group. This was therefore called the non-aggressive vc supergroup. In vc tests against representatives of the NAN and EAN aggressive supergroups, the non-aggressive supergroup was shown to be distinct from both of them. Frequencies of vc reactions against this supergroup, together with mating type data, are shown in Table 7.5. The isolates have been separated into two subsamples, the first from the relatively local area of Vermont, and the second made up of the remaining widely scattered isolates (see Table 7.1 and Figure 7.3). Results for the two subsamples were broadly similar, although data for those isolates incompatible with the supergroup have been combined due to the small numbers involved. In each subsample, both mating types were found among the supergroup, although the A type was predominant at 85 and 65% respectively. Compatible reactions between supergroup isolates are illustrated in Plate 7.3. Isolates giving fully incompatible reactions against the supergroup were all of different vc types, with the exception of four isolates from Manhattan, Kansas in the midwest, which were all of the same vc and mating type. Most (82%) of the isolates giving fully incompatible reactions against the supergroup were B mating types.

The supergroup was represented among isolates throughout the range of the sample, again with the exception of Kansas, as far apart as New Brunswick in southeast Canada, and North Carolina in the southeast U.S.A. The geographical distribution of the supergroup is illustrated in Figure 7.3.

Most incompatible reactions were clearly of the wide category, and although the features of other incompatible reactions varied between different pairs of isolates, no further categories, apart from the line-gap reaction, could be characterized. Mycelial penetration was measured in two experiments using fungicide tolerant isolates to confirm the vc structure of the sample and as a further attempt to recognise different reaction categories. Six pairings between isolates identified using morphological criteria as giving compatible or line-gap reactions against the supergroup all showed limited mycelial penetration (2-5 mm). This was not significantly different from control self pairings of ten isolates. Pairings against the supergroup of the 11 isolates initially identified with varying degrees of confidence as

Table 7.5. Frequencies of VC Reaction Categories against the North American Non-Aggressive Supergroup

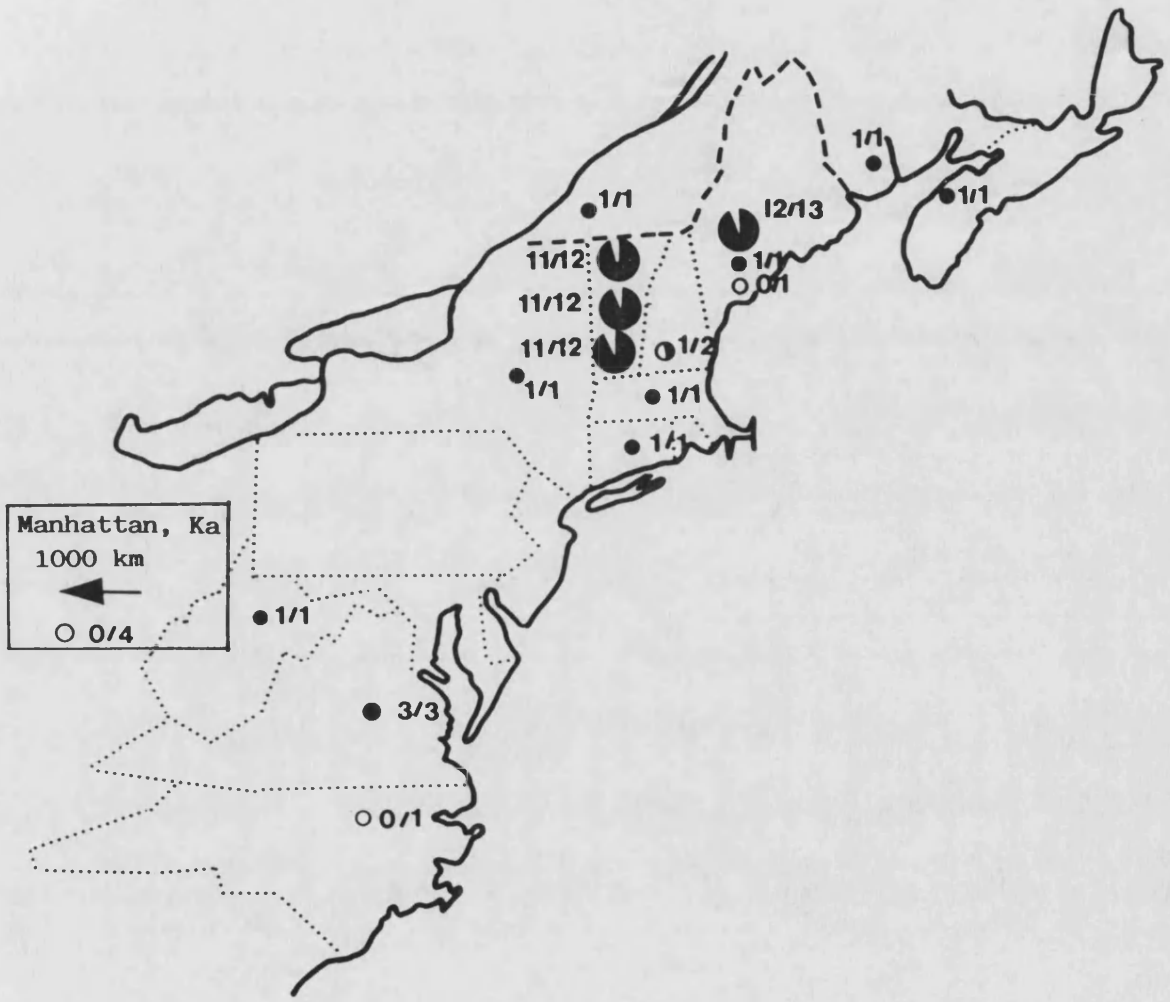
Sample	Sample size	Frequency of vc reaction category v. supergroup*		Total % A mating type
		% compatible+	% fully incompatible	
Vermont	36	92 (85)	16 (18)	78
Other states and provinces++	31	74 (65)		55

* % A mating type within each group shown in brackets.

\+ Includes one isolate giving a line-gap reaction against the supergroup.

++ Full details given in Table 7.1.

Figure 7.3 Distribution of the North American Sample



Circles show proportion of non-aggressive supergroup isolates (shaded) and non-supergroup isolates in each subsample.
Figures show number of supergroup isolates and subsample size.

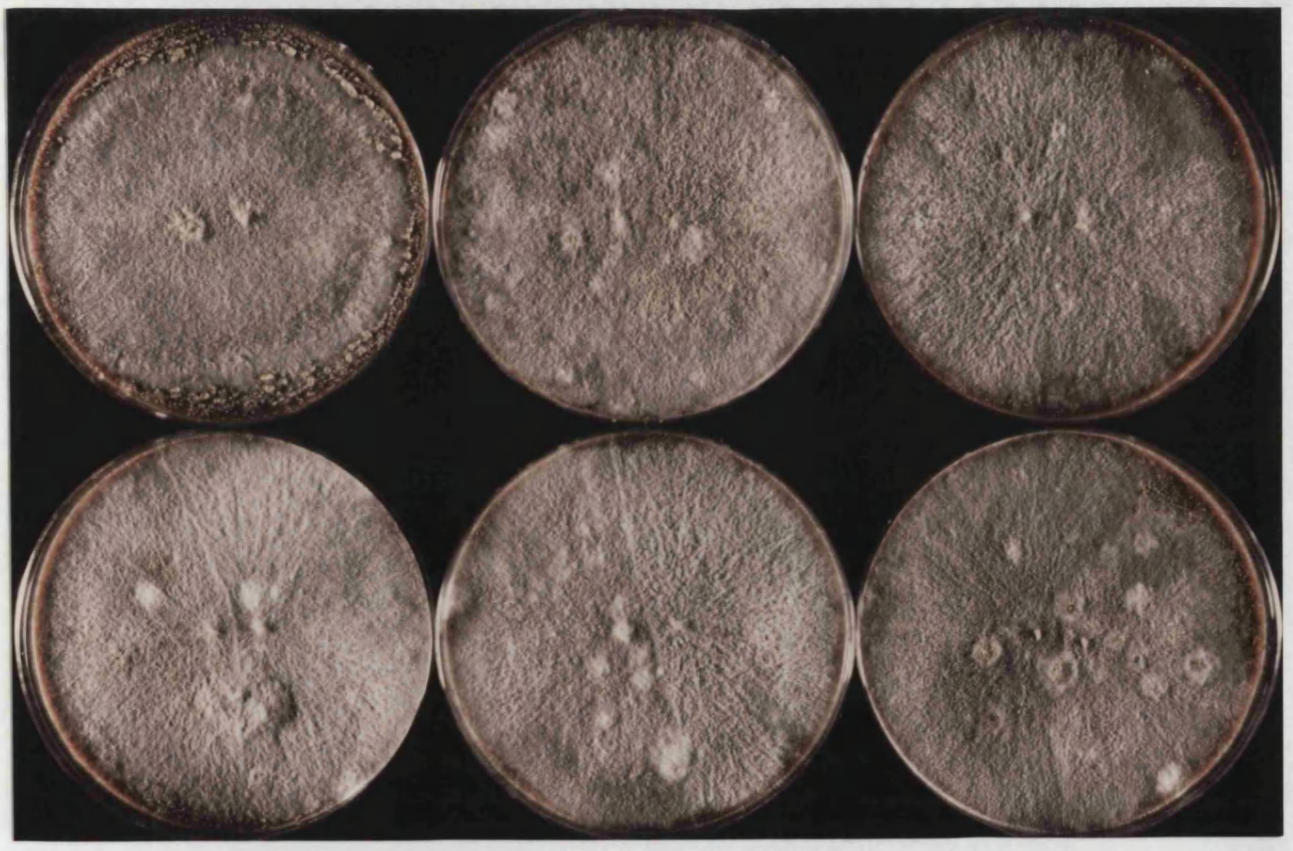
--- National boundary

····· State or province boundary

Scale 1:12500000

Plate 7.3 Compatible VC Reactions between North American Non-Aggressive Supergroup Isolates

A control compatible pairing of the same isolate is shown top left.



giving fully incompatible reactions showed differing extent and patterns of mycelial penetration, always to a greater depth than for compatible or line-gap reactions. Measurement of mycelial penetration in pairings within this group of 11 isolates confirmed the results of vc tests using wild-type fungicide sensitive isolates. Penetration between the four Manhattan, Kansas isolates was not significantly different from that in compatible reactions, and all pairings between a representative of the Manhattan isolates and the remaining seven isolates in all pairwise combinations showed greater mycelial penetration than compatible reactions.

The Spanish saprotrophic and pathogenic phase vc types detailed in Table 7.4 were vc tested against a representative of the North American non-aggressive supergroup. Three of them were found to give compatible reactions, one each from the Sancti Spiritus and Casa de Campo pathogenic phase samples, and one from CC-1, giving a frequency of 3% (3 out of 111 isolates) of the supergroup in the combined Spanish samples. Two of these three supergroup isolates were A mating types.

7.4 DISCUSSION

7.4.1 The Structure of Spanish Endemic and Epidemic Populations

The high level of heterogeneity for vc type, and near equal A and B mating type frequency in saprotrophic and pathogenic phase samples from both endemic and epidemic non-aggressive populations in Spain confirms the results of previous work with smaller European samples (Brasier, 1984). This strongly suggests a genetically varied and regularly outcrossing non-aggressive population. Since both endemic and epidemic populations were found to have the same structure, it is likely that the recent changes in selection pressure have not had any effect on the epidemic population, other than to greatly increase its size.

Underlying similarities with the Mersea Island NAN aggressive population, such as the mosaic population structure in bark and the low frequency of compatible reactions between pathogenic phase isolates, infer that there are essential similarities in the population biology of the two subgroups. It is therefore likely that the mosaic structure in the saprotrophic phase of the non-aggressive subgroup is initiated by the diversity of vc types carried by the vector beetles, and furthermore, that the diversity is maintained by the complex interactions between different genotypes thought to take place during the saprotrophic phase. Routine selection pressures acting at various points in the disease cycle are presumably the same for both subgroups.

Despite the lack of statistical difference between the mosaic pattern of colonistion in the endemic (Sancti Spiritus) and epidemic (Casa de Campo) saprotrophic phase samples (see Figures 7.1 and 7.2, and Table 7.3) it is probable that there are important differences which are not immediately apparent. The failure to reveal differences by statistical analysis is probably due to the sampling method and the limited number of vc types available for comparison. The uncertainties resulting from the gaps between the Sancti Spiritus grids and the different grid intervals used make it difficult to estimate the true area and extent of vc types. Since some vc types were probably also present in unsampled bark between the grids the areas occupied are very likely to have been underestimated, although the same argument could be applied to vc types extending beyond the limits of any grid. A more serious error results from approximation of the areas occupied by vc types in SS-D, which were isolated from a 20 mm interval grid, to 1 sq cm per isolation. However, this underestimate was thought to

give a more reasonable measure of the area occupied by each vc type than the considerable distortion resulting from approximation to 4 sq cm per isolation.

Taking into account the factors outlined above, it is probable that the vc types in the endemic samples do indeed occupy larger areas, and that there are consequently fewer vc types in a given area. This is may be explained by differences in disease levels and the consequent differences in the availability of breeding material at endemic and epidemic sites, with disease at the endemic Sancti Spiritus site barely noticeable (Plate 7.1), but with hundreds of dying trees at Casa de Campo. The greater flying distances for beetles in search of breeding material in endemic areas will increase mortality in the *O.ulmi* sporeload, especially in the hot, dry summers of the Spanish climate. The smaller size of *S.multistriatus*, which is a more important vector than *S.scolytus* in endemic areas (Webber, unpublished data) and consequent smaller spore loads (Webber & Brasier,1984; Webber, unpublished data), will increase the proportion of beetles arriving at breeding material without viable spores. In turn, fewer galleries will be inoculated with *O.ulmi*, although a fairly high proportion of occupied bark would still be expected as the result of secondary dispersal and the increased area for colonisation available to each vc type. This would explain the extensive, discontinuous areas occupied by each vc type, elongated along the grain as the result of faster growth in this direction, as seen in the Sancti Spiritus grids (Figure 7.2).

If bark is colonised by a smaller number of vc types occupying larger areas, then local diversity may be reduced as a consequence of more beetles carrying the same vc type. However, this will be compensated by increased dispersal distances, and factors reducing spore survival acting to greatly reduce the chances of one isolate encountering another of the same vc type. It has been suggested (Brasier,1986a) that ascospores might be better suited to adverse dispersal conditions. If this is the case, beetles are more likely to be still carrying ascospores when they arrive at breeding sites, contributing to the maintenance of heterogeneity.

The above arguments might predict greater similarity between the Casa de Campo non-aggressive and Mersea Island NAN aggressive epidemic samples. The large number of dying trees at Casa de Campo should make flying distances to breeding material comparable to those

on Mersea Island, and reduce spore mortality to a level below that for endemic populations. However, assuming that the large number of vc types in BHF-6 results from recolonisation and the establishment of recombinant genotypes, the relatively large areas occupied by vc types in both Spanish samples may result from continuous fungal activity throughout the shortened beetle breeding cycle of summer generations (perhaps as little as 6-8 weeks) due to increased temperatures. The beetle breeding in bark sampled from Sancti Spiritus and Casa de Campo in September and October would certainly have been at least the second generation of that summer. Absence of the long overwintering bark phase would make recolonisation unnecessary, and might also greatly reduce the opportunities for recombinant genotypes to colonise pupal chambers and contribute to beetle sporeloads. The role of ascospores may therefore be limited to primary dispersal on beetles, and not secondary or tertiary dispersal in bark.

7.4.2 Structure of the North American Epidemic Population

The discovery of a vc group at a very high frequency in the North American sample - the non-aggressive supergroup - in remarkable contrast to the level of heterogeneity in the Spanish population, supports the epidemic selection pressure model and provides important insights into the supergroup phenomenom. In accordance with the model, the presence of the supergroup can be explained by the ability of the non-aggressive to maintain an epidemic on the extremely susceptible American elm population. The selection pressures associated with epidemics are thought to favour the dominance of particular vc groups (Brasier, 1984, 1986a), as found in epidemic front populations of both aggressive subgroups.

The presence of the non-aggressive supergroup in the Spanish samples suggests that it could have been part of the population introduced to North America in the 1930s. Despite its present very low frequency in Spain it may well have made up a large proportion of the non-aggressive subgroup population assumed to have caused the European epidemics of the 1920s and '30s. Once an epidemic had been initiated in the highly susceptible American elm, the resulting episodic selection would have lead to the supergroup dominating the population. In Europe, the increasing importance of routine selection as epidemics declined would have reduced the supergroup frequency and favoured a more heterogeneous population. This effectively argues

against the high supergroup frequency in North America being a founder effect.

The presence of both A and B mating types in the supergroup indicates that it is not entirely genetically homogeneous. This illustrates an interesting point concerning supergroups, namely that their presumed high level of fitness in epidemic populations is likely to be directly associated with the vc loci themselves. It might otherwise be expected that episodic selection would lead to the development of several clonal vc groups. The mixture of mating types in the supergroup might be explained by the absence of sexual dimorphism in this subgroup, in contrast to the aggressive subgroups where the A mating type is adapted for increased sexual reproduction, and is less fit in the pathogenic phase than the B mating type. Any especially fit aggressive subgroup genotype is therefore likely to be B mating type, whereas in the non-aggressive mating type has not been shown to have any effect on fitness. However, the predominance of the A mating type in the non-aggressive supergroup suggests that perhaps it is at some advantage in the pathogenic phase. This might be detectable as differences in pathogenicity on American elm. Recent work by Brasier (unpublished data) has shown NAN supergroup isolates from epidemic front populations to be more pathogenic than isolates from the heterogeneous component.

If on the other hand, there is some advantage in maintaining the supergroup as mostly one mating type, the fungus may well have developed an alternative mechanism to bring this about. In the absence of differences in fitness associated with mating type this could be achieved by linkage of the vc and mating type loci, as found in the cross of H83O x H827. Most progeny of supergroup x non-supergroup matings carrying the parental vc genotype would therefore be of the same mating type as the parents. Since the cross of H83O x H827 also indicated that there is a relatively small number of vc loci functioning in the North American population, this would be particularly effective in ensuring that progeny of the supergroup vc genotype were mostly A mating type, without any need for differences in fitness to be conferred by the mating type alleles. However, the number of vc loci is obviously still sufficient to make most non-supergroup isolates of unique vc type.

Although the suggestion that only a small number of vc loci are functioning in the North American population is based on the results

of single cross (Table 6.3), some reduction in the number of vc loci might be expected. The uneven distribution of mating type between the supergroup and heterogeneous components of the population and the low frequency of isolates incompatible with the supergroup would mean that most wild matings will occur between supergroup and non-supergroup isolates. The progeny of such crosses are therefore likely to be isogenic with the supergroup at some vc loci, even if they are vegetatively incompatible. The existence of a supergroup in the North American non-aggressive population is supported by some earlier work on mating type distribution. Holmes (1958 and personal communication) found a very high frequency of A mating type isolates in Massachusetts, with the B mating type found in only 6 out of 112 towns and limited to the eastern part of the state. A mating type isolates were found in all of the towns sampled. From the evidence of more recent surveys of New England (Gibbs *et al.*, 1979; Houston, 1985), it can be assumed that only the non-aggressive was present in Massachusetts at this time. This strongly suggests that the non-aggressive supergroup was found at a higher frequency than in the present investigation.

Work by Shafer & Liming (1950), using a smaller sample from widely separated areas of the USA, showed a very interesting geographical distribution of the two mating types. Most of the isolates were A mating type, found throughout the sample area, including northeastern, southern, midwestern and northcentral states, and conformed to the description given by Walter (1937), now thought to be of the non-aggressive. The B mating type isolates were confined to Ohio and Indianapolis, and although similar morphologically to each other they were obviously different from the A mating type isolates. This can be interpreted as widespread distribution of the non-aggressive supergroup (A mating type isolates), and the appearance of the aggressive (B mating type isolates) in Indianapolis. Interestingly, only one of the 13 isolates from Indianapolis was an A mating type, suggesting replacement of the non-aggressive, and that the aggressive had therefore been present for several years. Shafer & Liming clearly recognised that the geographical distribution and morphological differences of the mating types suggested independent development of the Indianapolis infection area. The A mating type was more frequent in Ohio, the other state where it is proposed that the aggressive subgroup was present, implying that it

had appeared more recently.

Shafer & Liming described several of their isolates as behaving bisexually. Some with typical A mating type morphology behaved as B types in a few pairings against A types (named as Ab), and some with B type morphology behaved as A types in a few pairings against B types (aB). The geographical distribution of Ab isolates fitted with that of the A types, but aB isolates were restricted to Indianapolis and Ohio. Three further isolates were consistently bisexual, and mated with themselves. It is possible that these bisexual isolates were in fact pseudoselfing (Brasier & Gibbs, 1975b), to produce the missing mating type in each subgroup. The populations of both subgroups may have been at an early epidemic stage, with rapid asexual spread. The bisexual/pseudoselfing phenomenom may then have occurred in response to changing selection pressure acting against a uniform population structure, perhaps as the result of high levels of d-infection (Brasier, personal communication), and lead to a genetically more diverse population.

SECTION II - CONCLUSIONS

Characterization of non-aggressive subgroup vc reactions and confirmation of multigenic control allowed the vegetative compatibility system to be used in detailed investigation of the structure of saprotrophic and pathogenic phase populations. Bark was shown to be colonised by a mosaic of different vc types, occupying larger areas than those found in the NAN aggressive sample, BHF-6. This difference can probably be best explained by the effects of climate and the availability of beetle breeding material, rather than intrinsic differences between the aggressive and non-aggressive subgroups.

The highly heterogeneous population structure and near equal A and B mating type frequency among all of the Spanish samples is thought to be due to the dominant influence of routine selection pressures at endemic, low disease levels. The epidemic population has probably retained this structure because of its very recent derivation from the endemic population, despite the presumed influence of episodic selection in epidemics initiated by the NAN subgroup. The overall results infer that the same sorts of routine selection pressures may be acting on the Spanish non-aggressive and Mersea Island NAN populations, resulting in an essentially similar population biology and population structure.

It is suggested that the high frequency of a supergroup in the North American sample is due to the greater influence of episodic selection, leading to the dominance of a single vc type as a consequence of the extreme susceptibility of American elm and constant high level of disease and availability of beetle breeding material. The presence of the supergroup at very low levels in the Spanish samples supports the conclusion that the supergroup was probably part of the *O.ulmi* population introduced to North America from Europe in the 1930s. Since the supergroup is made up almost entirely of A mating type isolates, work from the 1950s can be used to support the proposal that the supergroup has comprised the majority of the North American non-aggressive subgroup population following its introduction, perhaps at higher levels than found in the present study.

SECTION III THE INTERACTION BETWEEN THE NAN AND NON-AGGRESSIVE SUBGROUPS

The history of the Dutch elm disease epidemics which have devastated the elm populations of much of the Northern hemisphere during this century appears to be closely concerned with changes in the pathogen itself. Significant events such as the appearance of the aggressive subgroups, the associated decline of the non-aggressive, and the separate but converging epidemics of the NAN and EAN subgroups in Europe (Brasier, 1986a, and described in Chapter 1), were not been recognised until their effects became obvious. Clearly, an understanding of the factors leading to such changes and any underlying mechanisms would be valuable for predicting future changes in the *O.ulmi* population. Insight into the factors contributing to the success of the aggressive subgroup and decline of the non-aggressive might allow any further changes to be directed towards a pathogen population more in balance with its host. In this context, an investigation of the processes involved in the replacement of the non-aggressive by the aggressive could be of fundamental importance.

The aggressive and non-aggressive subgroups can be considered to be adapted to virtually the same or closely overlapping ecological niches. Hence, when they have occurred together in contest for the same resource, the elm host, intense competition has resulted, leading apparently to strong selection against the non-aggressive. The barriers to the appearance of hybrids between these two major subgroups in wild populations has also prevented the emergence of new forms of *O.ulmi* on which selection could act.

Although these two major subgroups can be described as subspecies (on the basis of their reproductive isolation and differences for major characters under polygenic control (Brasier, 1982, 1983a, 1986a)), to formally describe them as such would fail to recognise the dynamic nature of the speciation processes which have lead to their origin. Even though the non-aggressive subgroup has now disappeared in many areas, selection in post-epidemic NAN and EAN aggressive populations may result in yet further changes in the pathogen. The outcome of these changes will largely determine the form of *O.ulmi* attacking future generations of elms, and may be particularly evident where large numbers of NAN x EAN hybrids are appearing in mixed populations of the two aggressive

races. Such phenomena of subgroups within fungal species have been increasingly recognised. Ecologically specialised subgroups, for example with different host ranges, have been described for many plant pathogens (reviewed by Brasier, 1987), and usually show some degree of reproductive isolation, even to the extent where they could be described as separate species. Although in many cases the subgroups have probably existed for some time but were unrecognised, some appear to be of more recent origin and may be examples of incipient speciation. Often this may be explained as a consequence of adaption to a new host following importation of either host or pathogen by man, or as a consequence of changing selection pressures acting on the fungus imposed by large scale monoculture of crop plants. The force behind such population changes has been described as an episodic selection event (Brasier, 1986a). Besides *O.ulmi*, perhaps the best examples of recent and presumably continuing changes resulting from man's influence are subgroups within Verticillium dahliae (Puhalla, 1979; Puhalla & Hummel, 1983) and Phytophthora megasperma (Hansen *et al.*, 1986). These ideas have been developed by Brasier (1987) to suggest that such sudden and intense selection will lead initially to a small number of pathogen genotypes (probably equivalent to vc groups) dominating the population. This may be associated with inbreeding or loss of the sexual stage and possibly followed in the longer term by genetic isolation and speciation.

Certainly, epidemiological studies of biotrophic pathogens of cereals, eg powdery mildew of barley (Wolfe, 1984) and rusts (Johnson, 1987), show that large scale monoculture of new varieties with resistance conferred by one or two genes rapidly selects a fungal population with the necessary virulence to overcome the host resistance. A similar situation may arise with fungicide tolerance conferred by mutation at a single locus, given the selective effects of widespread use of one group of fungicides. More stable control strategies are being developed using mixtures of resistant varieties and different fungicides, together with monitoring of the pathogen population to allow their predictive use (Wolfe, 1984; Georgopoulos, 1987; Skylakakis, 1987).

Clearly, population changes in such situations involve strong selection for only one or two genes, though with a critical influence on success. However, for necrotrophic fungi in which a single major selective force may be absent, fitness will be determined by many

factors influencing ability both to infect the host, and growth and subsequent sporulation utilising the killed tissue in competition with saprotrophes and other organisms. Thus, overall success will depend on a greater number of different characteristics, controlled by a correspondingly greater number of genes. In response to such a heterogeneous environment, a fungal population is itself likely to be more variable. It has been suggested that it may sometimes be partitioned into a more pathogenically fit component and a saprotrophic component, with pathogenic fitness maintained by feedback from the pathogenic phase and by recombination with the pathogenic phase (Brasier, 1986a). The complexity of the Dutch elm disease cycle suggests that success for any particular genotype of the pathogen will be determined by a large number of factors. Consequently, the interactions between the aggressive and non-aggressive subgroups will involve a broad range of these factors acting at many points in the disease cycle. Since the two major subgroups are adapted to occupy a similar niche, differences in the factors determining their relative success will probably be more quantitative than qualitative. Perhaps one notable exception to this is in their respective ability to produce cerato-ulmin, since the non-aggressive subgroup may not produce this toxin at all (Takai *et al.*, in preparation, Table 1.1). While cerato-ulmin production is clearly not an essential nor even the only determinant of pathogenicty, it may account for much of the difference in pathogenicity between these two subgroups.

Although the greater pathogenicity of the aggressive subgroup means that it can occupy a greater part of the xylem through killing more trees, this is not in itself sufficient to account for the rapid decline of the non-aggressive. Thus, in North America, where the non-aggressive alone has been able to cause a sustained epidemic, in competition with the aggressive it has still declined at a rate similar to that measured in Europe (Gibbs *et al.*, 1979; Houston, 1985; Brasier, 1986a).

The factors likely to be most important in determining the outcome of competition between the two subgroups were considered by Brasier (1986a) to be:

1. Pathogenicty. The greater pathogenicty of the aggressive subgroup would predict the occupation of a greater part of the xylem of a diseased tree, and would be important during early stages of the saprotrophic phase when overcoming residual host resistance

(Webber, 1979; Webber & Hedger, 1986).

2. Growth rate. A faster growth rate would increase the area of bark colonised, and would be of significance in beetle feeding grooves when establishing a mycelial phase from the initial spore inoculum.

3. Temperature relations. The different temperature optima for growth of the subgroups (Brasier *et al.*, 1981) will be important in determining relative growth rate. This implies an interaction with ambient temperature at critical points in the disease cycle, such as establishment in bark, the colonisation of pupal chambers, and transmission, and therefore also with climate.

4. Sexual fecundity. The greater fecundity of the aggressive subgroup *in vitro* would be expected to confer an advantage during the saprotrophic phase, influencing recombination of the pathogenic and bark phases and establishment of novel genotypes. Escape from cytoplasmic infection would also be increased.

5. Cytoplasmic infection. The transfer of d-factors between subgroups could be seen as 'biological warfare', since it might be expected that d-factors which have evolved with one subgroup may be more harmful to another.

6. Penetrating ability. The penetration effect, as a form of territorial invasion with its associated sporulation, could have considerable influence on area of bark occupied, secondary dispersal, and sporulation in pupal chambers during the saprotrophic phase. It might also be important in feeding grooves.

7. Barriers to hybridization. As discussed above, the fertility barrier between the aggressive and non-aggressive subgroups and poor fitness of hybrids would be expected to direct competition against the non-aggressive as the least fit subgroup.

8. Pathogenic feedback. The aggressive subgroup may be more able to grow out from the xylem into the bark and contribute to the sporeloads of the new generation of beetles, maintaining pathogenic and cytoplasmic fitness.

9. Rate of asexual spread. During epidemics a greater ability for rapid asexual dispersal, perhaps as a supergroup, would increase infection rates.

Several of these factors could themselves be further subdivided, and there will be interactions between them. For example, growth rate is a determinant of pathogenicity (Brasier & Webber, 1987); temperature relations have an effect on relative growth rate; and

pathogenicity itself has many determinants. The factors identified above are under polygenic control, and affect many different fitness parameters acting throughout the disease cycle.

The decline of the non-aggressive subgroup is unlikely to be due solely to an intrinsically lower impact during the disease cycle, but also to be a result of various mycelial interactions with the aggressive subgroup. Such competition is likely to be greatest during the long saprotrophic phase and in feeding grooves, where contact between different mycelia takes place. Indeed, the complex interactions which have been shown to take place during the saprotrophic phase, even within one subgroup, already suggest that competition between the aggresive and non-aggressive subgroups at this part of the disease cycle is likely to be of great influence on the overall *O.ulmi* population.

The outcome of competition in bark can be more easily studied and manipulated for experimentation than during other parts of the disease cycle. An investigation was therefore carried out on aspects of competition between the aggressive and non-aggressive subgroups during the saprotrophic phase, with the object of identifying factors involved in the decline of the non-aggressive.

Two main approaches were used. In the first, beetles carrying predominantly non-aggressive spores were introduced into the bark of trees with the xylem colonised by the aggressive, and *vice versa*, with a view to assessing the net outcome of competition during the saprotrophic phase. These experiments lasted for a considerable time and yielded a large but complex set of data. They influenced the direction of the second more controlled approach, looking in detail at critical aspects of competition between the aggressive and non-aggressive subgroups, both in culture and in elm bark.

8 OUTCOME OF AGGRESSIVE v NON-AGGRESSIVE COMPETITION IN SCOLYTID COLONISED BARK

8.1 INTRODUCTION

This part of the investigation attempted to measure the outcome of competition between the NAN aggressive and non-aggressive subgroups in the saprotrophic phase, in association with scolytid breeding. The most important measure of such competition will be the relative contribution of each subgroup to the sporeloads of the next generation of beetles, by comparison with their presence when the bark was first colonised. However, mixing the two subgroups in bark also gave an opportunity to look at other aspects of competition, including the pattern of colonisation of pupal chambers, and to a limited extent, changes in the proportion of each subgroup through the saprotrophic phase.

Since the experiments provided information on the range and proportions of aggressive and non-aggressive sporeloads on the emerging beetles, samples of these beetles were bred on to follow the fate of the two subgroups through to a second generation of beetles. An additional sample was allowed to feed on a healthy elm to study comparative colonisation of feeding grooves.

8.2 MATERIALS AND METHODS

A major difficulty in this investigation was that of creating a controlled mixture of the NAN aggressive and non-aggressive subgroups in bark. An ideal would have been to develop a source of beetles completely free of *O.ulmi*, coat them with a standardised sporeload, and allow them to breed in logs of a healthy elm. An attempt to surface sterilise pupae using a method described by Barras (1972), and breed on the resulting adults, failed to provide uncontaminated beetles in the subsequent generation.

An alternative reasonably controlled experimental procedure was required. It was thought that inoculating a healthy tree with isolates of one subgroup, and then allowing beetles carrying spores of the second subgroup to breed in logs from the tree would be suitable. However, an obvious disadvantage to this procedure was that although natural populations of beetles carrying NAN aggressive spores only were readily and widely available, it was no longer possible to have a source of beetles carrying only non-aggressive spores. The latter problem was partly overcome by inoculating trees with carbendazim tolerant NAN isolates, so that NAN isolates originating from the xylem could be distinguished from any wild-type isolates brought in by the breeding beetles along with the non-aggressive inoculum.

The investigation was carried out in two phases. The first was initiated in June 1984 using logs of a tree inoculated with isolates of the non-aggressive subgroup, and the second in June 1985 using logs of a tree inoculated with NAN *tol* isolates.

8.2.1 First Phase: Non-Aggressive Inoculated into Xylem with NAN Introduced on Beetles

A healthy English elm of 38 cm diameter breast height (dbh) at Eastbourne, East Sussex, supplied by kind permission of Eastbourne Borough Council, was inoculated on 21 and 22.6.84 with two non-aggressive isolates. The isolates, (H827 x H83O)F1-2 and (P82 x H365)F1-41, were single ascospore progeny of opposite mating type each with different parents and of different vc types. They had been shown to be free of dsRNA using polyacrylamide gel electrophoresis by Hilary Rogers, at Imperial College, London. Thus, the isolates were assumed to be free of d-infection, and any dsRNA found in non-aggressive isolates recovered from bark or beetles later in the experiment could be presumed to have originated from NAN isolates brought in by

the breeding beetles.

A dense yeast stage spore suspension resulting from c. 5 days incubation on a box shaker in Tchernoff's liquid medium was inoculated into chisel cuts at the base of the tree. Small squares of bark (c.2x2 cm) were removed using a mallet and chisel, taking care not to damage the xylem, with the lower horizontal cut slanted inwards to retain the inoculum. The xylem was quickly wetted with the inoculum and cut with the chisel, the aim being to maintain a constant supply of inoculum and prevent the entry of air into the xylem vessels. Initial uptake was rapid and could usually be maintained by deeper cutting of the xylem. These inoculations were made at 5-8 cm intervals around the circumference at two levels, one series alternating 6 cm above the other. Thus, much of the circumference was inoculated while retaining some xylem and phloem continuity. The two isolates were inoculated into alternate quarters of the circumference. An estimated total of 6OO ml of the spore suspensions was inoculated into the tree.

The tree was examined for disease symptoms on 2O.7.84, one month after inoculation. Only two or three branches in the upper crown showed symptoms, with a few twigs which had lost or carried dead leaves. A few epicormic shoots in the lower crown also showed some symptoms, but most branches remained healthy. The lack of fresh symptoms suggested that the disease was not spreading. Xylem samples were taken using a hollow punch at intervals up to 4 m above the inoculation points, and subsequent examination and re-isolation showed that the two non-aggressive isolates were in fact well distributed, despite the poor external symptoms.

The tree was felled on 26.7.84 by Eastbourne Borough Council, and the logs brought back to Alice Holt where the distribution and extent of xylem streaking was assessed visually from the presence of streaking and by re-isolation. Strong streaking was found in the springwood vessels of 1984, the year of inoculation, and also of the two preceeding years. At about 2.5 m above the inoculation points approximately 75% of the circumference of the 1984 and also the 1983 springwood vessels was heavily streaked, with less streaking in the 1982 vessels. A section of a branch from the crown showed steaking in only groups of the 1984 springwood vessels, totalling 1O-2O% of the circumference, although about 6O and 1OO% of the cicumference of the 1983 and 1982 vessels respectively were heavily

stained. Limited distribution in the 1984 annual ring may account for the poor symptom development. Subsequent isolations and vc tests showed that both of the inoculated isolates were well distributed in the tree.

The ends of the logs were sealed with Lac Balsam, and the logs taken to Barrow Hill Farm, Mersea Island on 31.7.84. They were left in hedgerows to attract beetles, as for the trap logs described in Section 2.1.2. The logs were recovered in October and left outside in a sheltered position until required. Some logs were left without beetle breeding in order to examine the ability of the non-aggressive isolates to grow into the bark from the xylem without assistance from breeding activity. Details of all the log treatments are summarised in Table 8.1.

Isolations were made at various stages of the investigation; from the bark over the winter before beetle emergence, from emerging beetles, and from pupal chambers after emergence. Isolations were also made from feeding grooves made by these beetles, and from their progeny after thay had been allowed to breed on in logs from a disease-free elm. Details of the procedures and isolation methods, essentially as described in Chapter 2, can be more conveniently included with the results and will not therefore be considered here.

8.2.2 Second Phase: Genetically Marked NAN Inoculated into Xylem with Non-Aggressive and some NAN Introduced on Beetles

Two apparently healthy smooth leaved elms of 28 and 23 cm dbh at Barrow Hill Farm (Trees 1 and 2) were inoculated on 25.6 and 9.7.85 with two NAN carbendazim tolerant isolates. The tolerant mutants had been selected from the wild-types HAY-51 and HAY-16 (A and B mating type respectively and of different vc types), isolated from diseased elm bark from Mersea Island in 1983. Inocula were prepared in Tchernoff's liquid medium, as described above.

Both Trees 1 and 2 took up only limited amounts of inoculum. To try and achieve distribution in the xylem comparable to that of the non-aggressive isolates in the Eastbourne tree, Tree 3, a small English elm at Alice Holt of 20 cm diameter at its base was similarly inoculated on 10.7.85. This tree rapidly took up large amounts of inoculum.

Tree 1 was inoculated by firstly removing small squares of bark as described for the Eastbourne tree. The springwood vessels tended

to come away with the piece of bark, damaging them and probably contributing to reduced uptake. This may have been due to late growth in the cool, wet spring and early summer of 1985, together with the apparently slower growth generally of trees at Barrow Hill Farm. Trees 2 and 3 were inoculated directly without removing the bark, using a 13 mm gouge chisel. Inoculum was run down the chisel as it was forced into the xylem, and a free flow achieved by moving the chisel downwards to a more horizontal position, and by lifting up the flap of bark above the chisel cut. The pattern of inoculation points described for the Eastbourne tree was used for all three trees, although the number of points depended on the circumference.

Neither of the Barrow Hill Farm trees (Trees 1 and 2) developed significant disease symptoms. One or two branches showed some yellowing and thinning of the foliage, and there were some epicormic shoots with dead leaves on the main trunk. Despite this, a limited field examination of streaking in twigs from the ends of larger branches suggested reasonable distribution. The Alice Holt English elm (Tree 3) rapidly developed severe symptoms over the whole crown.

When the trees had been felled they were brought back to Alice Holt and the extent and distribution of NAN *tol* isolates in the xylem assessed both visually from the presence of streaking, and by re-isolation. Streaking in logs from the Barrow Hill Farm trees was found to be relatively weak and poorly distributed. In Tree 1, streaking was seen in springwood vessels of each year from 1985 back to 1982. Limited isolations from the annual ring of each of these years all gave carbendazim sensitive NAN isolates, and it was concluded that much of the streaking was the result of previous infections, even though the tree was apparently healthy. Further isolations were made at 10 mm intervals around the circumference 2.5 m above the inoculation points (71 positions in total). Although the NAN was recovered from 41 positions, only 18 were NAN *tol* genotypes.

Tree 2 had moderate streaking in only the 1985 annual ring over most of the circumference up to c. 2 m, and well distributed but patchy streaking at c. 4m above the inoculation points. Isolations at 10 mm intervals around the cicumference at c. 4 m above the inoculation points (44 in total) gave 28 NAN *tol* isolates, and only one carbendazim sensitive NAN.

The Alice Holt English elm (Tree 3) had an intensity and distribution of streaking comparable to that of the Eastbourne tree inoculated with non-aggressive isolates in 1984. There was very strong streaking in the springwood vessels of 1985 only over the whole circumference up to 3m above the inoculation points. The trunks of Trees 1-3 were cut into suitably sized logs when required for experimental purposes, and the ends sealed with Lac Balsam. The beetles used to carry non-aggressive isolates into the bark of logs from Trees 1-3 were first generation laboratory bred progeny of Mersea Island *S.scolytus* from naturally infested material. They were collected and covered with spores of a range of non-aggressive isolates scraped from colonies growing on MEA, using a small paint brush. They were then allowed to breed in the experimental logs, contained by small mesh net bags. The logs were kept in the insectary at c. 20-30°C for several weeks until breeding was obviously well established, judging from the amount of frass, and then removed to a sheltered position outside for the winter.

Since these beetles had emerged carrying NAN spores before being covered with very large numbers of non-aggressive spores, detailed controls and checks were necessary. The situation was further complicated by variation in the intensity of streaking in logs from different trees (although in the end no isolations were made from beetles emerging from the logs of Tree 1). The following approach was used to assess the relative non-aggressive versus NAN inoculum levels of the beetles introduced, details of which are summarised in Table 8.1:

1. Beetles coated with non-aggressive spores, but also with a presumed natural inoculum of NAN spores, were allowed to breed in logs from Trees 2 and 3 (Logs 2/1 and 3/1 respectively).

2. Beetles with a natural inoculum of NAN spores only (ie untreated beetles) were allowed to breed in logs from Trees 2 and 3 (Logs 2/4 and 3/2 respectively).

3. Beetles coated with non-aggressive spores, plus a natural inoculum of NAN spores, were allowed to breed in logs of a disease-free English elm, Tree 4 (Logs 4/1, 4/2 and 4/3 respectively).

4. Ten *S.scolytus* which had been coated with non-aggressive spores were recovered from the logs after only 4-16 days, and isolations made from them using the dilution method (Chapter 2.1.3) to determine their relative load of NAN and non-aggressive spores. The results are summarised in Table 8.2. A surprisingly large range

Table 8.1 Summary of Source, Treatment and Isolations from Logs with Aggressive and Non-Aggressive Saprotrophic Phase Populations

Log no.		*O.ulmi* introduced		
		Via xylem inoculation	Via beetle breeding	Isolations carried out
9	English elm, Eastbourne	Non-aggressive, June 1984	Natural with NAN only, August 1984	Bark isolations before emergence
10	English elm, Eastbourne	Non-aggressive, June 1984	Natural with NAN only, August 1984	Bark isolations before emergence
11	English elm, Eastbourne	Non-aggressive, June 1984	Natural with NAN only, August 1984	a. Isolations from beetles in 1985 b. Isolations from pupal chambers in 1985 c. Source of beetles bred on to look at spores carried by progeny d. Source of beetles making feeding grooves
2/1	Smooth leaved elm, Barrow Hill Farm (Tree 2)	NAN *tol*, July 1985	Beetles carrying natural inoculum of NAN spores, coated with non-aggressive spores, August 1985	Isolations from late emerging beetles in 1986
2/4	Smooth leaved elm, Barrow Hill Farm (Tree 2)	NAN *tol*, July 1985	Untreated beetles, NAN only August 1985	Isolations from late emerging beetles in 1986
3/1	English elm, Alice Holt (Tree 3)	NAN *tol*, July 1985	Beetles carrying natural inoculum of NAN spores, coated with non-aggressive spores, August 1985	Isolations from peak emerging beetles and from pupal chambers in 1986
3/2	English elm, Alice Holt	NAN *tol*, July 1985	Untreated beetles, NAN only August 1985	Isolations from peak and late emerging beetles and from pupal chambers in 1986

was found in the number of non-aggressive spores carried. As expected these generally greatly outnumbered those of the NAN, although at the highest ratios of non-aggressive : NAN spores the presence of the NAN may sometimes have been missed on the isolation plates.

5. Isolations using the bark chip method (Chapter 2.1.2) were made in November 1985 from the bark of Log 4/3 (beetles coated with non-aggressive spores and bred in a disease-free log) to examine the establishment of the two subgroups in gallery systems. The results are summarised in Table 8.3. *O.ulmi* was isolated from all 34 galleries examined, and although both NAN and non-aggressive isolates were recovered from most galleries (68%), the non-aggressive was isolated much more frequently (67 v. 8%) from the 356 bark chips taken. The isolations from bark chips can be be considered as an indication of the relative areas occupied.

The results of isolations from beetles and bark suggested that the method had been successful in establishing the non-aggressive in bark.

Table 8.2 Results of Preliminary Isolations from S. scolytus Coated with Non-Aggressive Spores

Beetle no.	Days between introduction to log and sampling	Estimated no. of spores		% non-aggressive in sporeload
		NAN	non-aggressive	
1	4	0	1 495 000	100
2	4	5 500	228 500	98
3	4	0	1 335 000	100
4	4	6 000	44 500	88
5	4	0	45 000	100
6	4	1 500	270 000	99
7	15	27 500	23 500	46
8	16	3 000	22 500	88
9	16	50	50	50
10	12	0	52 000	100
mean		4 350	351 600	87

Table 8.3 Results of Isolations from Expanding Gallery Systems of *S. scolytus* Previously Coated with Non-Aggressive Spores

Total number of galleries examined	34
% galleries with non-aggressive only	32
% galleries with NAN only	0
% galleries with non-aggressive and NAN	68
Total number of bark chips	356
% bark chips giving non-aggressive only	54
% bark chips giving NAN only	8
% bark chips giving non-aggressive and NAN	13
% bark chips without *O.ulmi*	25

8.3 RESULTS

8.3.1 Isolations from Eastbourne English Elm Logs during the Winter

A series of isolations, mostly from 10 and 20 mm interval grids using chip and dilution methods (Chapter 2.1.2), was made during the autumn and winter between 7.11.84 and 26.2.85. Perithecia were frequently seen in the bark during the late autumn and early winter. Single ascospore progeny were isolated from perithecia using the method described in Chapter 2.5, although to release ascospores from perithecia which did not ooze they were squashed with watchmakers forceps rather than discarded. This increased the chances of successful isolations, but introduced a risk of isolating from maternal tissue of the perithecium.

To even out the growth rate differences between NAN and non-aggressive isolates the isolation plates were incubated at 25-27 ℃ whenever possible. Any *O.ulmi* growing on the plates was identified to subgroup after 5-7 days incubation, or at a later date from subcultures inoculated five per plate on MEA if a confident identification could not be made from the original plates. Most non-aggressive isolates were vc tested in 4x4 patterns against F1-2 and F1-41, the isolates originally inoculated into the tree, using the method described in Chapter 2.3.2, in order to identify them as one or other of the original isolates or as a recombinant progeny genotype.

The results of these isolations are summarised in Table 8.4. F1-2 and F1-41 were recovered from the bark whenever isolations were made. NAN isolates were recovered more frequently than non-aggressive isolates throughout the period in which isolations were made. The isolations from nine perithecia tended to follow this pattern and although 'NAN' and 'non-aggressive' perithecia were found in the same mother gallery, no hybrid perithecia were found.

Isolations were also made from the bark of control logs in which beetles had not been allowed to breed. Small squares of bark were removed with a mallet and flamed chisel, and chip isolations made from the exposed surface of the xylem, the exposed (cambial) surface of the inner bark, and the inner bark after cutting away the exposed surface. The non-aggressive was recovered from a fairly high proportion of the xylem chips (59%), but from only 15% of the bark chips (Table 8.4).

8.3.2 Isolations from Emerging Beetles

Logs were placed in fine mesh net bags net bags prior to emergence to

Table 8.4 Results of Isolations from the Bark of the Eastbourne English Elm during Autumn and Winter

Date	Stage of colonisation	Isolation method	Results
7.11.84	Log 10. Discrete unsuccessful *S.scolytus* galleries	Chip isolations from 3 gallery systems, and dilution isolations from 3 systems	Non-aggressive isolates F1-2 and F1-41 recovered from 2 galleries using chip method. F1-2 and F1-41 recovered from all 3 galleries using dilution method; NAN predominant in one
23.11.84	Log 9. Established *S.scolytus* galleries	10mm interval grid over 7 galleries. Total area 172 sq.cm. Additional dilution isolations from 10 grid positions. Isolations from perithecia	70% of chips gave NAN only. 0.5% of chips gave non-aggressive only. 0.5% of chips gave both. All non-aggressive isolates F1-41. Of 9 perithecia from 2 galleries 8 were NAN and 1 non-aggressive
14.12.84	As above	Mixed 10 and 20mm interval grid over 9 galleries. Total area 308 sq. cm, 156 chips	58% of chips gave NAN only. 12% of chips non-aggressive only. 2% of chips gave both
25. 2.85	As above	20mm grid over 8 galleries. Total area 508 sq. cm, 127 chips. Chip isolations from an additional 10 galleries	94% of chips gave NAN only. 4% of chips gave non-aggressive only. 6 galleries gave NAN only. 1 gallery gave non-aggressive only. 3 galleries gave both. All non-aggressive isolates F1-41
	Controls		
8. 1.85	Control log without breeding	Two bark chips from each of 10 randomly chosen points	No. *O.ulmi* isolated
29. 4.85	As above	Xylem and bark chips from each of 34 points, as described in text	Non-aggressive isolated from 59% of xylem chips and 15% of bark chips

prevent beetles escaping. The emerging *S.scolytus* were collected at intervals. Isolations were made to estimate their total sporeloads and the proportions of the NAN and non-aggressive subgroups using the dilution method (Chapter 2.1.3).

Only one log from the Eastbourne English elm inoculated with non-aggressive isolates in 1984, Log 11, provided sufficient beetles for isolations. Emerging beetles were collected from 5-27 June '85. Counts of NAN and non-aggressive colonies were made after a convenient period of incubation at 25-27 °C , usually 4-6 days. Five non-aggressive colonies were then chosen at random from each beetle found to be carrying non-aggressive spores and vc tested against F1-2 and F1-41. Isolations were made from beetles emerging from logs of Trees 1-3 originally xylem inoculated with carbendazim tolerant NAN isolates in 1985, and from logs of disease-free Tree 4, from 21 June - 21 July '86. Counts were made of the numbers of beetles emerging each day to provide a record of the pattern of emergence.

In the earliest isolations a random sample of 25 NAN colonies from each beetle were tested for carbendazim tolerance (not necessary for Log 4/1) by subculturing onto MEA + O.5 ppm carbendazim and assessing growth after 3-5 days. The isolation method was later modified to include a duplicate set of dilutions on MEA + O.5 ppm carbendazim. The number of replicate plates was reduced to three for each set of $1x10^{1}$ dilutions and two each for subsequent dilutions. Both methods allowed the numbers of non-aggressive, NAN wild-type and NAN *tol* spores on each beetle to be estimated. Representative NAN *tol* isolates from beetles emerging from Logs 3/1 and 3/2 were vc tested in 4x4 patterns against the isolates originally inoculated into the trees, HAY-51 and HAY-16, to identify any recombinant genotypes. The results of isolations from beetles in both 1985 and 1986 were complicated by two additional interacting variables. Firstly, the total sporeload and the relative contribution of isolates inoculated into the xylem (the pathogenic phase) depended on the point in the overall period of emergence at which the beetles were collected. Beetles emerging after the main peak of emergence (post-peak emerging beetles) were found to have both greater mean sporeloads, and to carry a larger proportion of isolates originating from the pathogenic phase. It is assumed that there is a gradual change in these factors over the emergence period. Consequently, the differences in 'earlier' and

'later' emerging beetles were not statistically significant for all samples, depending on the portion of the emergence period during which the beetles in each sample were collected. Since this situation was only revealed gradually as isolations from the second part of the investigation were underway in 1986, there is only sufficient comparative data for some of the logs. However, it was possible to re-examine the data from the first part of the investigation in 1985 (Log 11), and it then became apparent that the duration of the emergence period depended on weather conditions.

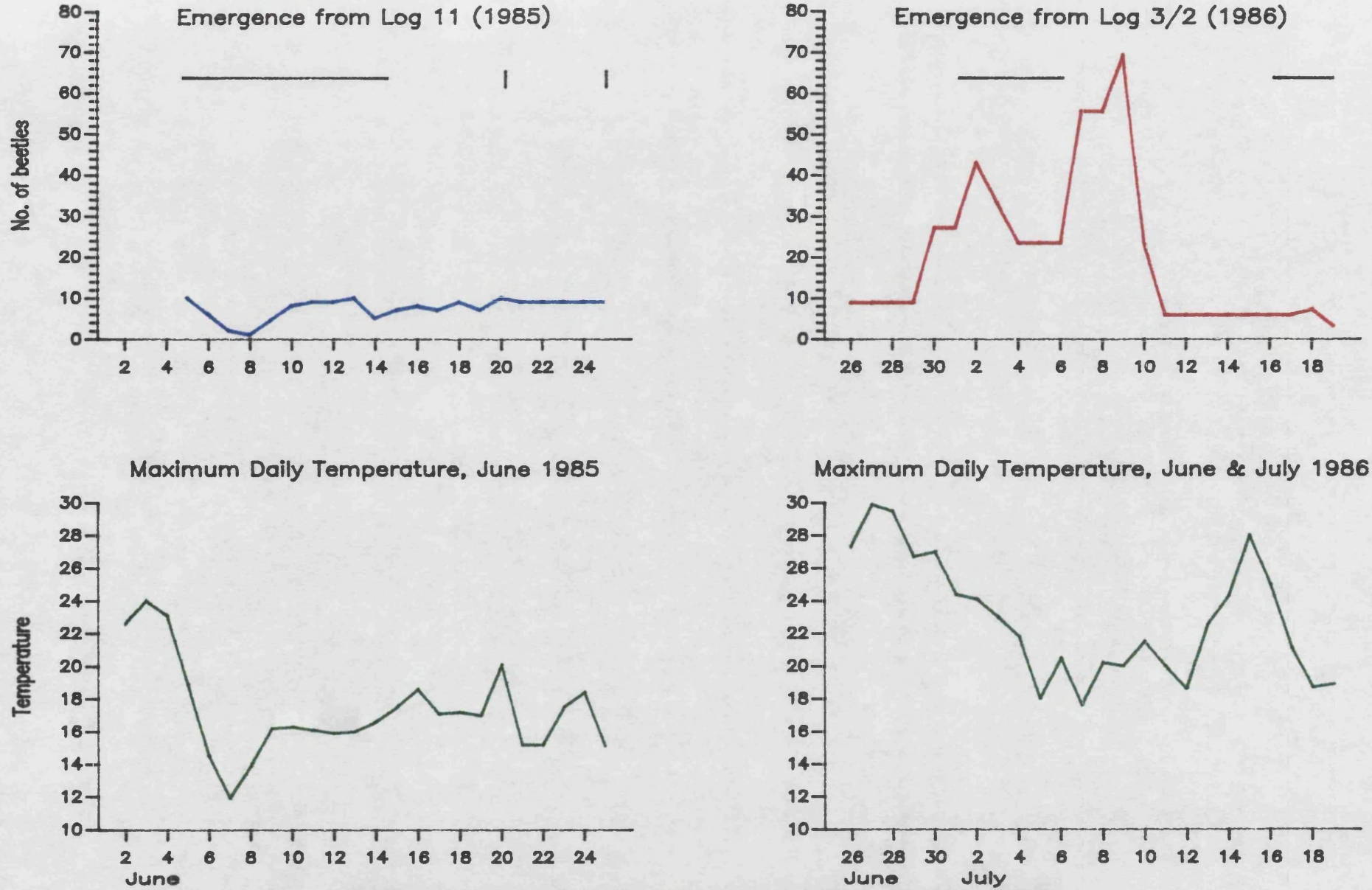
Figure 8.1 shows the maximum daily temperature during the period of beetle emergence in 1985 and 1986, and the daily counts of beetles emerging from Log 3/2. For Log 11 and Log 3/2 the days on which beetles were collected for isolations have been shown. Bearing in mind that the temperature threshold for flight is c. 20°C , it is not surprising that beetles emerged slowly from Log 11 in 1985, and did not show any peaks of emergence. In contrast, emergence from Log 3/2 in 1986 developed very rapidly immediately after a period of hot weather, and followed a long period of cooler weather in June which had delayed emergence. Emergence then dropped off very rapidly, despite continuing warm weather.

Figure 8.2 shows the sporeloads of beetles emerging from Log 3/2, and the proportion of spores derived from the pathogenic phase. The sample has been divided into beetles collected during the early part of the peak of emergence, and those collected late in the emergence period, as shown in Figure 8.1. Both the sporeloads and the proportion of xylem-derived inoculum of post-peak emerging beetles were significantly larger ($P<0.001$) than those of beetles collected in the early part of the peak period of emergence.

The numbers of beetles emerging each day from Logs 2/1, 2/4, 3/1, 3/2 and 4/1 are shown in Figure 8.3, together with the days on which beetles were collected. Complete data are only available for Logs 3/1 and 3/2. General observation of the numbers of beetles emerging from the other logs, and the similarities of the later part of the graph with the data for Logs 3/1 and 3/2, are sufficient to divide samples taken from them as representing either peak or post-peak emergence.

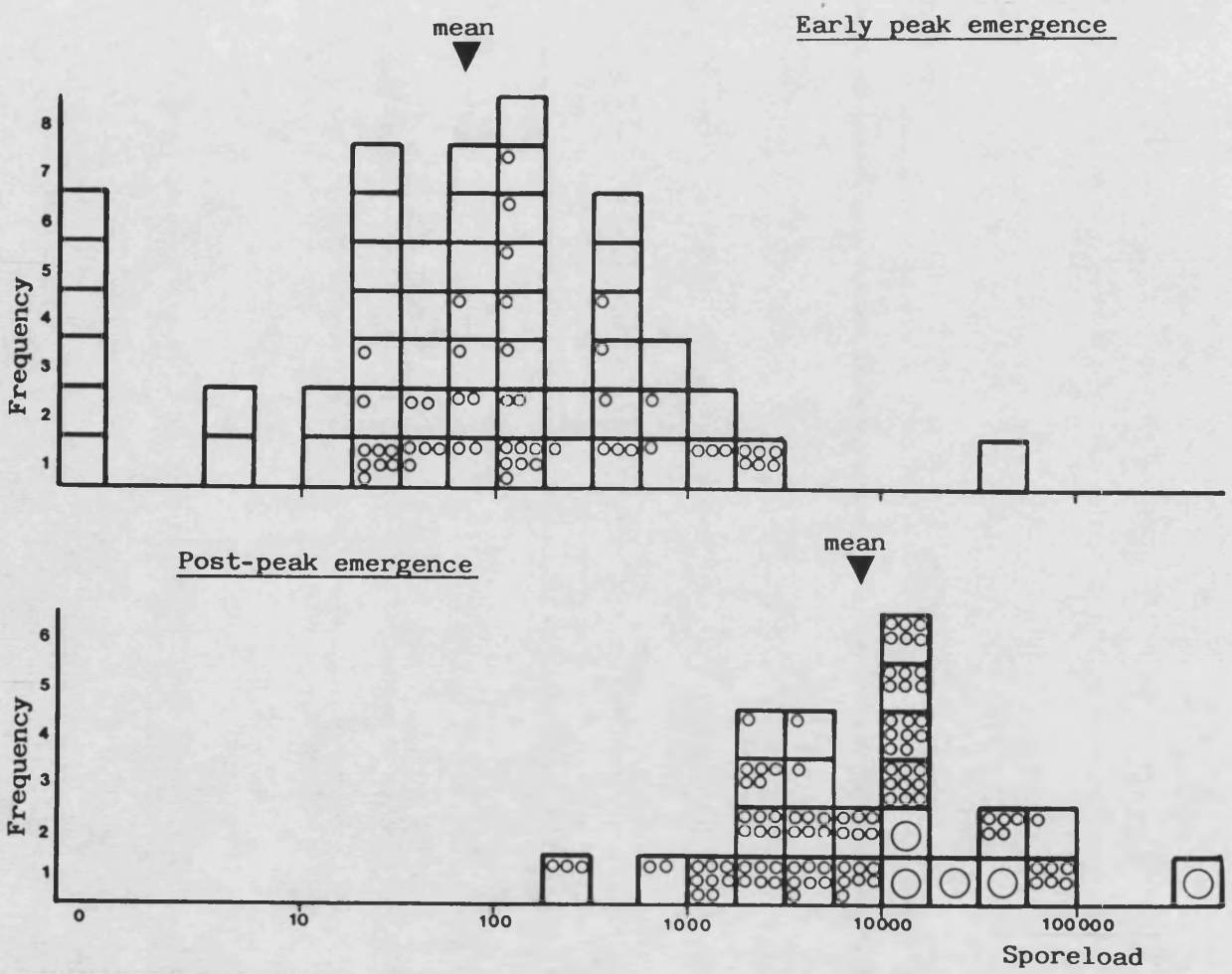
Figure 8.4 shows the total sporeloads and the proportions of non-aggressive, NAN, and NAN *tol* spores as appropriate, in the sporeloads. Data for Logs 11, 3/1 and 3/2 are the combined results of isolations from early peak, peak or post-peak emerging beetles as shown

Figure 8.1 Emergence from Logs 11 and 3/2, and Maximum Daily Temperatures during the Emergence Period



Horizontal lines and vertical bars show beetle sampling dates.

Figure 8.2 Sporeloads of Beetles Sampled from Log 3/2 during Early Peak and Post Peak Emergence



Circles show the proportion of NAN tol (xylem-derived inoculum) in the sporeload.
A small circle represents 10% of the sporeload. A large circle represents > 90% of the sporeload.

Figure 8.3 Number of Beetles Emerging per Day and Sampling Times for Logs used in 1986

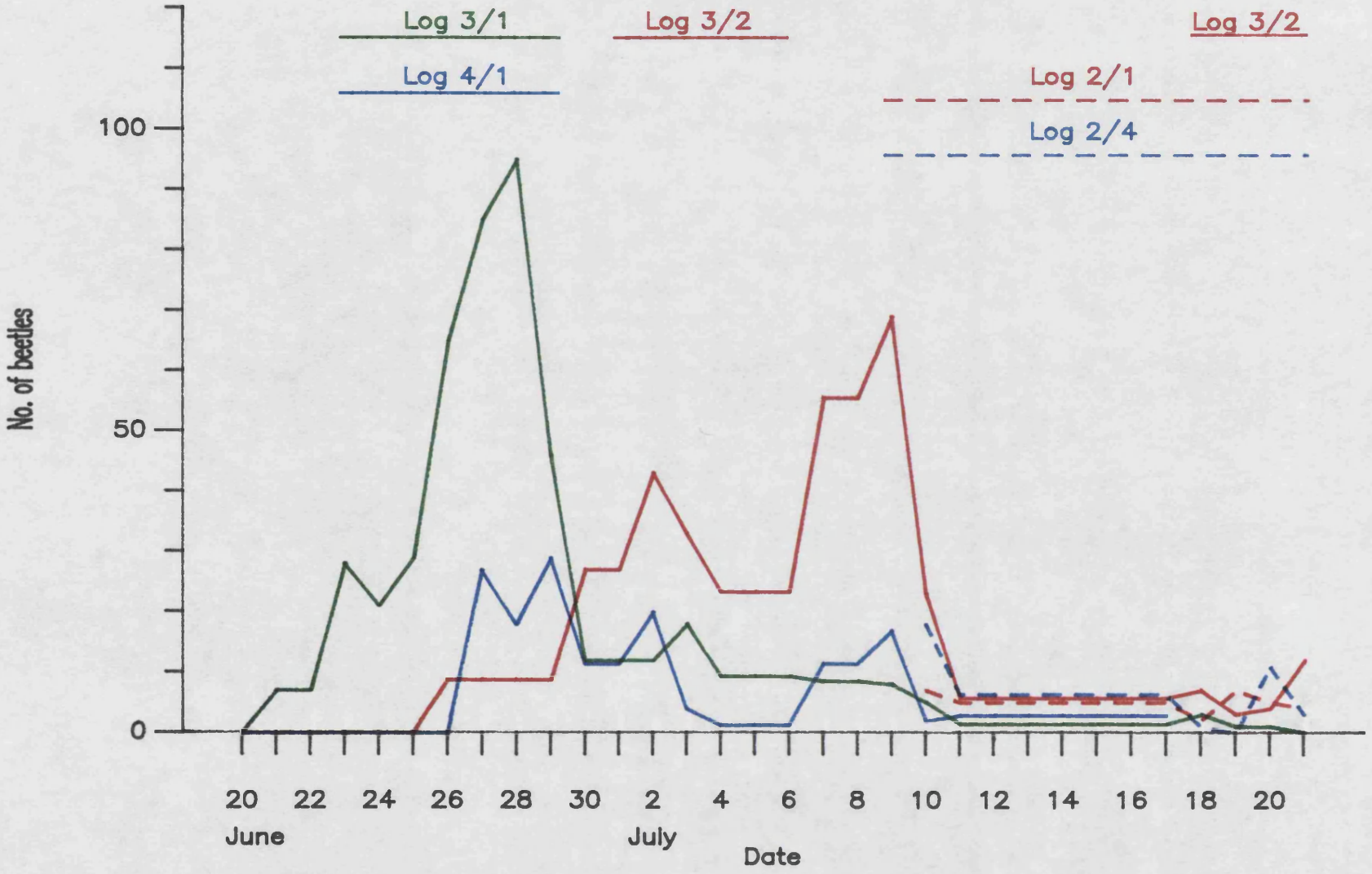
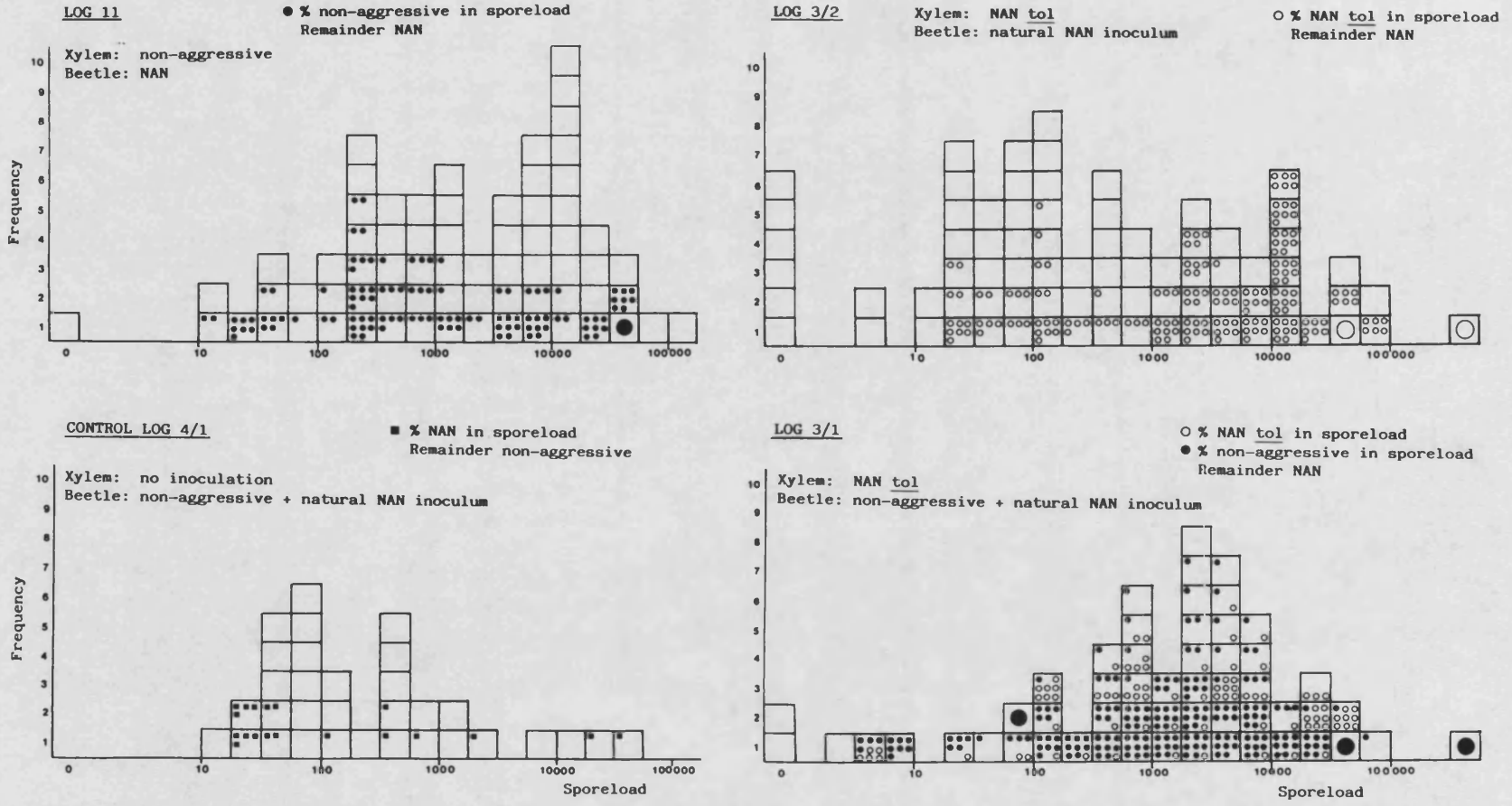


Figure 8.4 Sporeloads of Beetles Sampled from Logs 11, 3/1, 3/2 and 4/1 - Combined Data for the Whole Emergence Period



A small circle or square represents 10% of the sporeload.
A large circle represents >90% of the sporeload.

in Figures 8.1 and 8.3. Data for Log 4/1 are from peak emerging beetles only, but since there was no pathogenic phase involved in Tree 4, the time of emergence would probably have affected only the total sporeload, and not the proportion of NAN and non-aggressive spores in the sporeload. Data for Logs 2/1 and 2/4 have not been presented in this form, but followed the same pattern and are summarised in Table 8.5 (see below).

The extreme variations in relative sporeloads considerably complicates the overall interpretation of the data and emphasises the need for large samples. It may be helpful to compare isolations from beetles from different logs on the basis of the mean sporeloads, and the mean percent recovery of each spore type (non-aggressive, NAN or NAN *tol*), bearing mind the variation within the samples. The mean percent recovery was calculated as the overall sample mean for the percent of each spore type per beetle, and does not therefore take any account of the size of the sporeload. The data are summarised in Table 8.5. The results show that later emerging beetles tended to have greater spore loads, and tended to have a greater proportion of spores derived from the pathogenic phase. Isolates from the emerging beetles which had been identified as originating from the xylem were screened for vc type against the isolates originally inoculated into the xylem. This tested for evidence of sexual recombination between the isolates introduced into the xylem, or between xylem isolates and wild-type isolates introduced on the beetles. The results are summarised in Table 8.6. It should be noted that all non-aggressive isolates on beetles from Log 11 must be either one of those inoculated into the xylem, or their progeny. For Logs 3/1 and 3/2, NAN *tol* isolates may be either one of those inoculated into the xylem, their progeny or *tol* progeny of matings between one or other of the NAN *tol* isolates inoculated into the xylem and NAN wild-type isolates brought into the bark by the beetles. It was not possible to separate the last two categories, and for the latter case half of the progeny would have been carbendazim sensitive and therefore unidentifiable as recombinants. The data have been expressed as the percent of the original or recombinant isolates among the xylem-derived isolates tested. However, nearly all of the beetles from which the xylem-derived isolates came also carried other genotypes, namely NAN wild-type on Log 11 and Log 3/2 beetles, and NAN wild-type and non-aggressive on Log 3/1 beetles.

Recombinants were identified among the isolates from beetles

Table 8.5 Summary of the Results of Isolations from Emerging Beetles

Log	Original log inoculation: Via xylem	Via beetles	Beetle Sampling time*	No. of beetles sampled	Mean beetle sporeload	Mean % recovery of each subgroup: non-aggressive	NAN wild-type	NAN tol
11	non-aggressive	NAN	'peak'	49	8 100} 9 600	14} 17	86} 83	-
			'post-peak'	20	13 500}	25}	75}	-
Log 11, progeny forced 1985	none	NAN and non-aggressive (Log 11 beetles)	unknown	25	42 000	<1		-
Log 11 progeny forced 1986	none	as above	unknown	43	100	7	93	-
2/1	NAN tol	non-aggressive and some NAN	post-peak	26	30 500	22	73	5
2/4	NAN tol	NAN	post-peak	24	28 500	-	78	22
3/1	NAN tol	non-aggressive and some NAN	peak	50	12 700} 14 400	48} 45	38} 42	14} 13
			post-peak	5	31 100}	5}	62}	33}
3/2	NAN tol	NAN	early peak	52	1 100} 10 300	-	89} 71	11} 28
			post-peak	25	29 400}		41}	59}
Control								
4/1	none	non-aggressive and some NAN	'peak'	34	1 500	96	4	-

* Peak = Beetles sampled when large numgers of beetles were emerging.
Post-peak = Beetles sampled after most beetles had emerged. Classification uncertain for Log 11 beetles.

Table 8.6 Estimated Frequencies of Recombinants between Xylem Inoculated Isolates among Isolates Recovered from Beetles

			% among isolates of xylem origin recovered from beetles		
			Original isolates		
LOG 11	No. of isolates tested	No. of beetles	F1-2 type	F1-41 type	Recombinant type non-aggressive
'peak'	113	31	23	59	18
'post-peak'	73	18	45	44	11
LOG 3/1			H16 tol type	H51 tol type	Recombinant type NAN tol
peak	181	33	1	38	61
post-peak	32	4	0	84	16
LOG 3/2					
peak	56	13	0	41	59
post-peak	148	25	0	74	26

Peak = beetles sampled when large numbers of beetles were emerging.
Post-peak = beetles sampled after most beetles had emerged.

from all three logs, but a higher proportion of recombinants was found on the earlier emerging beetles. However, beetles from Logs 3/1 and 3/2 also carried a much higher frequency of (NAN) recombinants than those beetles from Log 11 (non-aggressive). It should be remembered that the NAN *tol* isolates in the xylem of Tree 3 would also have been able to mate with wild-type NAN isolates in the bark, whereas the non-aggressive isolates from the xylem of the Eastbourne tree would only have been able to mate with each other. A higher frequency of recombinant isolates in the former might therefore be predicted.

8.3.3 Isolations from Pupal Chambers after Beetle Emergence

Isolations from pupal chambers in Log 11 were made from 12-30 July '85, when all the beetles had emerged. They were made from pupal chambers in three different positions:

i. Partly in the inner bark and scoring the xylem.

ii. In the inner bark but only touching the xylem.

iii. In the inner and/or outer bark entirely away from the xylem.

Pupal chambers were firstly examined for *O.ulmi* sporulation. Stab isolations were made from any synnemata, and some isolations made from perithecia as described above. Isolations were made from approximately equal numbers of pupal chambers in each position, 44 in total, mostly using the chip method with 5-10 chips per pupal chamber. Some pupal chambers were brushed with a wetted, sterile sable brush, which was then rinsed in 5 ml of sterile water and isolations made from a dilution series. The resulting colonies were identified to subgroup as described above.

To estimate the relative proportions of pupal chambers made in different positions within the bark, 100 exit holes chosen at random were followed back to pupal chambers and their positions recorded. Counts were also made of the positions of all pupal chambers in one bark piece from which isolations had been made, making a combined total of 156 pupal chambers. Six different positions were recognised, including those from which isolations were made:

i. Pupal cells in the xylem only.

ii. In the inner bark and scoring the xylem.

iii. In the inner bark but touching the xylem.

iv. In the inner bark away from the xylem.

v. Across the inner and outer bark away from the xylem.

vi. In the outer bark only.

Isolations were made from c. 60 pupal chambers from each of Logs 3/1 and 3/2 between 30 July and 3 August '86. Approximately equal numbers of pupal chambers were examined from each of the following positions:

i. In the inner bark and scoring the xylem.

ii. In the inner bark away from the xylem.

iii. In the outer bark.

Any *O.ulmi* sporulation was noted. All isolations were made using the chip method, taking 5 or 6 chips from each pupal chamber. The resulting isolations were identified to subgroup and all NAN isolates were tested for carbendazim tolerance. The proportion of pupal chambers in each position was estimated by examining nearly all pupal chambers in randomly selected bark pieces, a total of over 220 for each log.

The results of isolations from pupal chambers in Logs 11, 3/1 and 3/2 are summarised in Tables 8.7 and 8.8. Presentation as the percent of pupal chambers in each position giving each subgroup or isolate type (Table 8.7) takes no account of the proportion of each type in the pupal chambers, only their presence or absence. Presentation as percent of the total number of chip isolations (Table 8.8) emphasises differences in the proportion of each subgroup and isolate type occupying pupal chambers in different positions within the bark.

As might be expected, the results showed a general trend of decreasing frequency of isolates from the xylem in pupal chambers further away from the xylem, and a corresponding increase in isolates brought in by the beetles. However, a fairly high but variable proportion of pupal chambers not in contact with the xylem gave a mixture of isolates originating from xylem and beetles.

Data for different isolation methods used for Log 11 pupal chambers were insufficient to allow detailed comparison. However, they suggested that chip isolations are more likely to show both subgroups to be present, but that dilution isolations may give a better measure of the proportions of each, especially in terms of spore numbers. It was not possible to make many stab isolations from synnemata and perithecia in the pupal chambers, because by the time isolations were made the spore blobs were either missing or had dried up. However, their physical state suggests that both synnemata and perithecia were present before and during beetle emergence.

The subgroup types of 19 perithecia sampled from Log 11 pupal

Table 8.7 Isolations from Pupal Chambers in Logs 11, 3/1 and 3/2 - as Percent of Pupal Chambers with Isolates from each Original Inoculum Source

Position of pupal chambers	No. of pupal chambers examined	% of pupal chambers giving isolates originating from: *			
		Xylem only	Bark only	Xylem and bark	% without *O.ulmi*
LOG 11 (non-aggressive in xylem, NAN on beetles)					
Inner bark, scoring xylem	12	8	0	92	0
Inner bark, touching xylem	17	6	6	88	0
Inner and outer bark, away from xylem	15	0	13	87	0
LOG 3/1 (NAN *tol* in xylem, non-aggressive and some NAN on beetles)					
Inner bark, scoring xylem	20	0	65	35	0
Inner bark, away from xylem	20	0	80	20	0
Outer bark	22	0	59	35	5
LOG 3/2 (NAN *tol* in xylem, NAN on beetles)					
Inner bark, scoring xylem	19	10	37	53	0
Inner bark, away from xylem	21	5	67	24	5
Outer bark	20	5	70	10	15

* The actual subgroup or isolate type from each source depended on the isolates originally inoculated into the xylem or carried into the bark by the beetles, as detailed in Table 8.1

Table 8.8 Isolations from Pupal Chambers in Logs 11, 3/1 and 3/2 - as Percent of Bark Chips giving Isolates from each Original Inoculum Source

Position of pupal chambers	No. of pupal chambers examined	Total no. of bark chips	% of bark chips giving isolates originating from: *			
			Xylem only	Bark only	Xylem and bark	% without *O.ulmi*
LOG 11 (non-aggressive in xylem, NAN on beetles)						
Inner bark scoring xylem	12	88	27	27	36	5
Inner bark touching xylem	17	101	12	38	47	3
Inner bark and outer bark away from xylem	15	132	11	56	26	7
LOG 3/1 (NAN *tol* in xylem, non-aggressive and some NAN on beetles)						
Inner bark, scoring xylem	20	115	3	54	2	33
Inner bark, away from xylem	20	104	10	61	6	14
Outer bark	22	104	6	52	10	32
LOG 3/2 (NAN *tol* in xylem, NAN on beetles)						
Inner bark, scoring xylem	19	114	31	65	0+	4
Inner bark, away from xylem	21	105	11	72	0	17
Outer bark	20	100	4	54	0	42

* The actual subgroup or isolate type from each source depended on the isolates originally inoculated into the tree, carried into the bark by the beetles, as detailed in Table 8.1.

+ Chips giving both NAN wild-type (from bark) and NAN *tol* isolates (from xylem) are unlikely to have been detected.

chambers are shown in Table 8.9. Most of the perithecia examined (14) were from matings between NAN isolates, and the remainder were of the non-aggressive subgroup. Although some pupal chambers contained both NAN and non-aggressive perithecia, no hybrid perithecia were identified. Non-aggressive perithecia were not found in pupal chambers away from the xylem.

The visual assessment of *O.ulmi* sporulation in pupal chambers in Logs 11, 3/1 and 3/2 is summarised in Table 8.10. It is likely that some sporulation present at the time of beetle emergence had disappeared by the time assessments were made. Most pupal chambers in all three logs in contact with the xylem contained either synnemata, perithecia or both. The frequency of fruiting structures in pupal chambers away from the xylem was low, and no sporulation was seen in pupal chambers entirely within the outer bark (Logs 3/1 and 3/2).

The frequencies of pupal chambers in different positions within the bark of Logs 11, 3/1 and 3/2 are shown in Table 8.11. There were considerable differences between Log 11, and Logs 3/1 and 3/2. In Log 11 virtually no pupal chambers were formed in the outer bark, but this position accounted for about half of the pupal chambers in Logs 3/1 and 3/2. The frequency of pupal chambers in contact with the xylem was correspondingly higher in Log 11 (27%) compared to Logs 3/1 and 3/2 (14 and 12%).

8.3.4 Isolations from Further Generations of Log 11 Beetles

Some of the beetles emerging from Log 11 were collected and allowed to breed in disease-free logs. One set of beetles began breeding in an English elm log on 7.6.85. The log was maintained at c. 20-30℃ in an insectary and isolations made from 25 of the next generation of adults using the standard method when they began to emerge on 7.8.85. Single chip isolations were made from c. 50 pupal chambers, and three chip isolations made from each mother gallery found when all the bark was removed from the log. Resulting colonies were identified to subgroup.

A second set of beetles emerging from Log 11 was allowed to breed in disease-free smooth leaved elm logs from Barrow Hill Farm. These were left outside in a sheltered position until 27.3.86 when they were transferred to the insectary at c. 20-30℃ . Beetles began to emerge on 15.4.86. Isolations were made from 43 beetles, and the resulting colonies identified to subgroup.

Table 8.9 Subgroup Types of Perithecia from Log 11 Pupal Chambers

Position of Pupal chamber	No. of pupal chambers examined	No. of perithecia yielding: NAN genotypes only	No. of perithecia yielding: Non-aggressive genotypes only
Inner bark, scoring xylem	2	1	2
Inner bark, touching xylem	4	9	3
Inner and outer bark, away from xylem	2	4	0

Table 8.10 *O.ulmi* Sporulation in Pupal Chambers in Logs 11, 3/1 and 3/2

Position of pupal chambers	No. of pupal chambers examined	% of pupal chambers with each type of sporulation				
		Synnemata only	Perithecia only	Synnemata and perithecia	Mycelium only	No fruiting structures
LOG 11 (non-aggressive in xylem, NAN on beetles)						
Inner bark scoring xylem } Inner bark, touching xylem}	18	44	0	6	22	28
Inner and outer bark away from xylem	37	16	8	8	32	35
LOG 3/1 (NAN *tol* in xylem, non-aggressive and some NAN on beetles)						
Inner bark, scoring xylem	20	25	15	40	0	20
Inner bark, touching xylem	20	10	0	0	0	90
Outer bark	22	0	0	0	0	100
LOG 3/2 (NAN *tol* in xylem, NAN on beetles)						
Inner bark, scoring xylem	19	26	21	11	5	37
Inner bark, touching xylem	21	5	0	0	5	90
Outer bark	20	0	0	0	0	100

Table 8.11 Positions of Pupal Chambers in Logs 11, 3/1 and 3/2

	LOG 11	LOG 3/1	LOG 3/2
No. of pupal chambers examined	156	281	222
Position of pupal chambers	% of pupal chambers in each position		
Pupal cell in xylem Inner bark, scoring xylem Inner bark, touching xylem	27	14	12
Inner bark, away from xylem	51	20	17
Across inner and outer bark	21	23	18
Outer bark	1	43	53

The sporeloads of the two sets of second generation beetles are shown in Figure 8.5, and summarised in Table 8.5. Sporeloads of the progeny of the first set of beetles, forced to complete the breeding cycle in the same season, were extremely high. However, only one non-aggressive colony was seen on the isolation plates of the 25 progeny. In contrast, sporeloads of the progeny of the second set of beetles, forced to emerge in early spring 1986, were exceptionally low, but gave a slightly higher (c. 7%) frequency of non-aggressive isolates.

Only the NAN aggressive was isolated from maternal galleries and pupal chambers in the bark of the English elm log containing the first set of beetles. From a total of 123 chip isolations, three from every maternal gallery found in the bark, 57% gave the NAN subgroup. The remainder did not yield any *O.ulmi*. 70% of single chip isolations from each of 50 pupal chambers chosen at random also gave the NAN, and the remainder did not yield any *O.ulmi*. It should be noted that the log was maintained at 20-30℃ , and that the breeding cycle was completed in only c. 9 weeks.

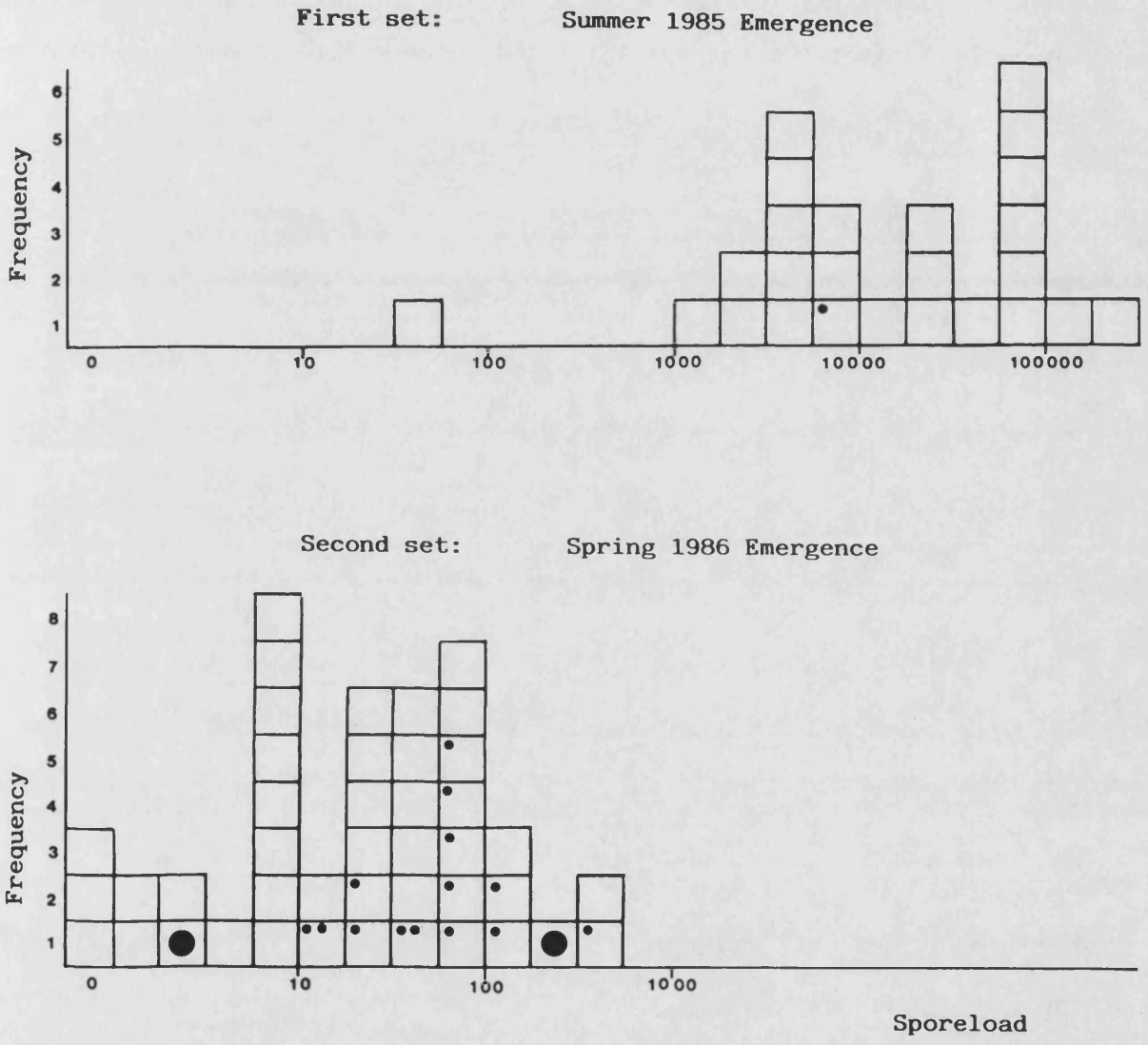
8.3.5 Isolations from Feeding Grooves made by Log 11 Beetles

Further samples of the beetles emerging from Log 11 were caged on a small potted English elm, using a fine mesh net bag. Initially, 30 *S.scolytus* were introduced to the elm on 20.6.85, and an additional 35 on 27.6.85. Twig crotches were examined for feeding grooves at intervals from 28 June to 13 August '85, and different coloured map pins used to mark feeding grooves made since the previous inspection, as described by Webber & Kirby (1983). Isolations were made from 25 feeding grooves and associated xylem streaking above the grooves on 6.9.85, and from a further 25 on 24.9.85, using mostly the chip method. Some isolations were made from a dilution series after brushing feeding grooves with a wet sable brush, as described for pupal chamber isolations. The resulting colonies were identified to subgroup after appropriate incubation.

Isolations from the feeding grooves are summarised in Table 8.12. *O.ulmi* was recovered from 72% of feeding grooves, but only 2% gave non-aggressive isolates only. A fairly high proportion (22%) of feeding grooves had associated xylem infection, 20% by NAN isolates.

A comparison .of chip and dilution isolations from the same feeding grooves did not reveal any major differences between the results of the two methods. Six feeding grooves yielded the same subgroups with both methods. One which give NAN only with chip isolations

Figure 8.5 Sporeloads of Further Generations of Log 11 Beetles



Circles show the proportion of non-aggressive in the sporeload.
A small circle represents 10% of the sporeload.
A large circle represents > 90% of the sporeload.

Table 8.12 Summary of Results of Isolations from Feeding Grooves made by Beetles From Log 11

No. of feeding grooves examined	50	50
Subgroup(s) isolated	% of feeding grooves	% of associated xylem infection
No. O.ulmi obtained	28	78
NAN only	36	20
Non-aggressive only	2	2
NAN and non-aggressive	16	0

also gave low numbers of non-aggressive colonies on the dilution plates in addition to NAN colonies. Three feeding grooves from which no *O.ulmi* was isolated by the chip method gave very low numbers of NAN colonies on the dilution plates.

Some isolations from strong streaking yielded NAN isolates and indicated a small number of successful infections. All of the NAN xylem isolates were therefore vc tested in 4x4 patterns (Chapter 2.3.1), on the assumption that infections originating fron different feeding grooves were likely to have been caused by different vc types. The number of different vc types was then considered to represent the number of successful infections.

8.4 DISCUSSION

The experiments provided much interesting and useful information on many aspects of the saprotrophic phase. However, the results are not easy to interpret with respect to competition between the aggressive and non-aggressive subgroups.

The relative contributions of the xylem and bark inocula originally introduced into the logs to the sporeloads of emerging beetles were shown to be influenced by a number of variables. In particular, the weather before and during the emergence period, the positions of the pupal chambers in the bark, and differences in the origin and quality of *O.ulmi* sporulation in pupal chambers according to their position, all affected the composition of the beetle sporeloads. A better understanding of the interaction and role of these variables may help predict the factors likely to be important in competition between the two subgroups. However, it will be more convenient to discuss firstly the results of more general significance to events taking place during the saprotrophic phase, and then to consider the signifance for competition between the aggressive and non-aggressive subgroups.

8.4.1 General Significance of the Results

In the last few years it has become apparent that the relationship between the vector beetles and *O.ulmi* has a considerable influence on the spread of Dutch elm disease (Webber & Brasier, 1984; Webber, 1987). The importance of variation in the sporeloads of individual beetles and the high threshold number of spores needed for successful infection have been discussed previously (Chapter 1). The strong relationship between time of emergence and size of sporeload reported here (Tables 8.7, 8.8 and 8.10), can be explained on the basis of the earlier emergence of beetles from pupal chambers in the outer bark (Fransen, 1939; Beaver, 1967), where conditions are less favourable for colonisation and sporulation of *O.ulmi*. Webber & Brasier (1984) have previously recorded greater sporulation in pupal chambers in the inner bark. Thus, sporeloads will tend to be greater on beetles emerging later from pupal chambers in the inner bark, where a higher moisture and nutrient content is likely to lead to the formation of more synnemata and perithecia. In addition, pupal chambers touching or scoring the xylem are more likely to be colonised by isolates located in the xylem. Such isolates may produce synnemata and so contribute directly to the sporeloads of beetles without strictly colonising the

bark.

By inference, later emerging beetles will tend to come from pupal chambers in the inner bark, and will tend to have greater sporeloads and a higher proportion of genotypes from the xylem. The results do also show that both isolates from the xylem and recombinants between genotypes originating in the pathogenic and saprotrophic phases can colonise pupal chambers well away from the xylem.

The proportion of beetles carrying large numbers of spores may therefore be determined by the distribution of pupal chambers in the bark. Data for the postions of pupal chambers in Logs 11, 3/1 and 3/2 showed considerable differences in this respect. Very few pupal chambers were formed entirely within the outer bark of Log 11, whereas almost half of the pupal chambers in Logs 3/1 and 3/2 were. This may have influenced the total sporeloads and contribution from the pathogenic phase, although possibly because of the enormous variation between individual beetles (Figure 8.4) no such differences were detected statistically (Table 8.5). The factors determining the positions of pupal chambers are unclear, but winter temperature, and thickness and nutrient status of the inner and outer bark are likely to be involved.

The timing of beetle emergence relative to the reduction in susceptibility of elms as dependence on springwood vessels decreases through the summer (Parker *et al.*, 1941), may have a considerable influence on the resulting level of infection. Differences in weather from year to year will affect the pattern of emergence, as found in 1985 and 1986 (Figures 8.1 and 8.3), and in a year when emergence is delayed by relatively cold temperatures, elms may be less susceptible when significant numbers of beetles do eventually emerge and disperse. Conversely, if a period of hot weather occurs early in the season, flight may take place when the elms are at their most susceptible. The length of the emergence period, and the relative timing of the peak of emergence, will also be important.

8.4.2 Significance of the Results for Aggressive v Non-Aggressive Competition

Bearing in mind the factors described above, several observations can be made regarding competition between the NAN and non-aggressive subgroups. Isolations made over the winter from the bark of logs from the Eastbourne tree inoculated in the xylem with non-aggressive

isolates (Table 8.4), showed that the non-aggressive was able to successfully colonise bark from the xylem in competition with NAN isolates. Levels of the non-aggressive subgroup in bark were variable but generally low, with no evidence of a gradual increase in feedback from the pathogenic phase as found by Webber & Brasier (1984) with an NAN isolate in the xylem. Similar levels of bark colonisation by non-aggressive isolates from the xylem were found in the xylem of inoculated logs without beetle breeding (Table 8.4). However, the apparent advantages of colonising bark unoccupied by other isolates may be balanced by the lack of breeding activities of beetles which might otherwise facilitate release of the fungus from the xylem.

The results of re-isolations from beetles coated with non-aggressive spores (Table 8.2) showed that the technique used to initiate the saprotrophic phase with a high proportion of non-aggressive inoculum was relatively successful. Thus, very large numbers of non-aggressive spores were isolated from beetles recaptured after only 4 days with the logs, and fairly high numbers after 12-16 days. Moreover, isolations from galleries made by these beetles (Table 8.3) confirmed the predominant establishment of the non-aggressive in bark, although the NAN was also present in a high proportion of galleries tested (Table 8.3). Taking into account the results of isolations from individual bark chips, it is estimated that the non-aggressive occupied about five times as much bark as the NAN.

Isolations from the subsequent generations of beetles emerging from logs of the Eastbourne tree in summer 1985, and Trees 2 and 3 in summer 1986, suggested that isolates introduced into the xylem made similar contributions to beetle sporeloads (Table 8.5) regardless of the subgroup involved. However, direct comparisons between the various experiments were hard to make because of difficulties in estimating the time in the emergence period at which beetles were collected, and the effect of the increasing proportion of xylem-derived inoculum in sporeloads as emergence proceeded. The data for beetles sampled during the peak of the emergence period are likely to be more reliable for such comparisons because of the larger sample sizes and greater total numbers of emerging beetles. For peak emerging beetles the mean percent recovery of the xylem isolates from beetles was; 14% non-aggressive for Log 11, 14% NAN *tol* for Log 3/1 and 11% NAN *tol* for Log 3/2, and on late emerging beetles 25, 33 and 59% respectively (Table 8.5). There were therefore no large or

apparent differences in the abilities of the non-aggressive and NAN *tol* isolates to feed back from the pathogenic to the saprotrophic phase and contribute to the sporeloads of the next generation of beetles.

The results may also be considered in terms of the number of beetles from which only isolates originating from the bark were recovered, illustrated in Figure 8.4. Again, intepretation is complicated by the interaction of the time during emergence when beetles were sampled, and the greater proportion of pathogenic phase isolates in the sporeloads of late emerging beetles. For peak emergence, 35% of beetles from Log 11 carried the NAN only, 31% from Log 3/1 carried NAN wild-type and non-aggressive only, and 43% from Log 3/2 carried NAN wild-type only. For late emerging beetles the data are 10, 20 and 0% respectively. Thus, the data do not show any differences in the success of NAN or non-aggressive isolates introduced into the bark by the breeding beetles to contribute to the sporeloads of emerging beetles.

The situation is rather more interesting when other factors are taken into consideration. The presence of NAN *tol* isolates in the xylem had a major influence on the ability of low levels of wild-type NAN brought in by the breeding beetles relative to levels of non-aggressive on the beetles to compete with the non-aggressive during the saprotrophic phase. The proportion of wild-type NAN spores in the sporeloads of beetles emerging from Log 4/1, which had no *O.ulmi* in the xylem, was very low when compared to the proportion on beetles emerging from Logs 3/1 and 3/2, with NAN *tol* in the xylem and non-aggressive plus some wild-type NAN or wild-type NAN only on the breeding beetles (Table 8.5 and Figure 8.4). The data are 4, 38 and 89 mean percent recovery of wild-type NAN on peak emerging beetles from Logs 4/1, 3/1 and 3/2 respectively.

Thus it appears that the presence of NAN *tol* isolates in the xylem allowed the low level of wild-type NAN brought in by the beetles to compete more successfully with the initially much higher levels of non-aggressive. Competition may have begun during the first stages of bark colonisation in beetle breeding galleries, where beetles usually score the xylem and probably facilitate the entry of xylem isolates to the bark. The process is then likely to have continued throughout the saprotrophic phase.

The reasons for this synergism between NAN isolates brought in by the beetles and those present in the xylem are unclear. It may be an

effect of recombination between the pathogenic and saprotrophic phases, or due to some unrecognised aspect of aggressive versus non-aggressive competition. The size of the effect suggests that further investigation would be worthwhile.

The higher levels of wild-type NAN recovered from beetles emerging from Logs 3/1 and 3/2 may be partly explained by considering the proportion of isolates arising via recombination between those inoculated into the xylem. In Log 11, the recombinant non-aggressive isolates detected could only have arisen as a result of mating between F1-2 and F1-41. In Logs 3/1 and 3/2 the recombinant NAN *tol* isolates detected may have arisen either from mating between the two original NAN *tol* isolates, in which case all the progeny will be NAN *tol*, or matings between the original NAN *tol* isolates and wild-type isolates brought in by the breeding beetles. In the latter case, only half of the progeny will be NAN *tol* and the remainder will be unidentifiable as the products of recombination between the pathogenic and saprotrophic phases. The degree of feedback from the pathogenic phase may therefore have been underestimated in Logs 3/1 and 3/2. The results presented in Table 8.6 show a higher proportion of recombinant isolates on beetles emerging from Logs 3/1 and 3/2 compared to Log 11. However, it is possible that a similar increase in feedback from the pathogenic phase might have occurred with the non-aggressive subgroup, if for example low levels of non-aggressive spores had been present on the beetles initiating the saprotrophic phase in Log 11.

The results of isolations from pupal chambers suggested that although the non-aggressive subgroup was able to colonise most pupal chambers regardless of their position in bark (Table 8.7), it was unable to make a significant contribution to the sporeloads of beetle emerging from those pupal chambers (see Figure 8.4). In general, F1-2 and F1-41 were recovered more frequently from pupal chambers touching or scoring the xylem than from pupal chambers entirely away from the xylem (Tables 8.7 and 8.8). The reverse was true for isolates brought in by the breeding beetles. The results for pupal chambers in Log 3/1 do not follow this pattern very closely, possibly because the sampling was not detailed enough given the involvement of three isolate types, NAN *tol* from the xylem and wild-type NAN and non-aggressive from the bark.

Isolations made from perithecia found in the bark of Log 11

before emergence (Table 8.4) and from pupal chambers after emergence showed that the two non-aggressive isolates were both able to contribute to the saprotrophic phase and to produce perithecia. Since recombinant genotypes were isolated from the bark as well as from beetles, it also appears that secondary dispersal of the resulting ascospores occurred, and lead to the establishment of novel genotypes in the bark. Some of the recombinant non-aggressive isolates recovered from beetles, however, are likely to have been from ascospores picked up directly from perithecia in pupal chambers.

No NAN aggressive x non-aggressive hybrids were isolated at any stage of the investigation. This was despite the fact that a very large number of separate isolations from bark chips and beetles, probably totalling tens of thousands, were examined during the course of the investigation, and despite intimate mixing of the two subgroups, as illustrated by the large numbers of pupal chambers and beetles from which both subgroups were recovered. This provides further evidence that the two subgroups do not produce fit hybrids in nature. Interpretation of the results of isolations from the further generations of beetles emerging from Log 11 which had been bred on in logs from disease free elms, was complicated by the large differences in sporeloads of beetles emerging from the two logs involved (Figure 8.5 and Table 8.5).

This extreme variation in sporeloads was probably due to the unusual conditions, such that the summer 1985 beetles would have completed a generation in minimum time, and the spring 1986 beetles may have emerged before *O.ulmi* was able to colonise the pupal chambers following the winter. Assuming that the proportions of NAN and non-aggressive spores were unaffected by the variations in sporeloads, then the level of the non-aggressive subgroup would have undergone a reduction relative to that carried in by the Log 11 beetles.

The recovery of NAN and non-aggressive isolates from feeding grooves made by beetles emerging from Log 11 (Table 8.12) corresponded reasonably well with the number of Log 11 beetles found to be carrying non-aggressive spores, and the proportions of NAN and non-aggressive spores in their sporeloads (Figure 8.4 and Table 8.5). Although competition between aggressive and non-aggressive mycelia in the feeding grooves would be expected, it is difficult to comment on the relative success of xylem infection by each subgroup, owing to the wide variation in total sporeloads, in the proportions of

the two subgroups in the sporeloads, and the relationship of these factors to the threshold spore numbers required for infection.

9 INVESTIGATION OF CRITICAL ASPECTS OF COMPETITION

9.1 INTRODUCTION

The factors potentially involved in competition beween the aggressive and non-aggressive subgroups, as discussed in the introduction to this Section, were reconsidered in the light of the results presented in Chapter 8. Those thought to be closely concerned with initial establishment in bark and with mycelial interactions throughout the saprotrophic phase were chosen for further investigation.

The experiments carried out in Chapter 8, beginning with one subgroup in the xylem and the other predominant on the breeding beetles, readily produced a saprotrophic phase composed of both subgroups. This in turn resulted in many of the subsequent generation of beetles carrying spores of both subgroups. A similar structure to the sporeloads of beetles could be predicted to occur in wild populations of *O.ulmi* comprising both aggressive and non-aggressive subgroups.

In such circumstances, the two subgroups would be in direct competition with each other from the very beginning of the saprotrophic phase, ie when establishing from a mixture of spores in fresh beetle galleries in dying elm bark. Relative germination and growth rates are likely to be key factors in determining the outcome of competition at this stage, and these in turn may be influenced by temperature and the relative ability to overcome residual host resistance.

If the two subgroups occur together in bark (cf Brasier & Gibbs, 1976; Lea, 1977; and Section II), then the results of interactions between competing and developing mycelial colonies will also be important in determining the area of bark occupied. A considerable amount of evidence (Lea, 1977; Webber & Brasier, 1984; and Sections I and II) shows that saprotrophic phase populations are dynamic, with changes within the population of a single subgroup taking place during the saprotrophic phase. This evidence also strongly suggests that the changes result from competition between different mycelia. Here the penetration effect is likely to be a key aspect of such competition. This too may be influenced by growth rate and temperature, as well as by differences in intrinsic penetrating ability.

Ultimately, the area of bark occupied by any particular subgroup or genotype will play an important part in the eventual colonisation of pupal chambers. Furthermore, the penetration effect between mycelia within pupal chambers may result in and is certainly likely to influence sporulation and the relative contribution of various

competing genotypes to the sporeloads of beetles.

To summarise, of the factors discussed in the introduction to this Section, those that are likely to be particularly involved with initial establishment and mycelial interactions during the saprotrophic phase are; relative pathogenicity towards bark with residual host resistance, growth rate, temperature relations, and penetrating ability. The experiments described in this Chapter were designed to investigate the contributions of these factors to competition between the aggressive subgroups, and more specifically the role of:

1. Competitive establishment from mixed spore inocula.
2. Relative penetration between opposed isolates.
3. The influence of residual host resistance on growth in bark.

Potential interactions between the above factors meant that two or more variables had to be taken into account in any one experiment, in particular the likely interactions between temperature and growth rate. While the effects of residual host resistance could only be studied in elm bark, other experiments were extended from work in culture to elm bark wherever possible.

9.2 MATERIALS AND METHODS

Although the same basic methods were used throughout each part of the investigation, some modifications were made to subsequent experiments repeating and expanding preliminary work. Particular difficulties were experienced with some of the experiments in bark, due to problems in developing suitable methods and the generally higher level of variation and uncertainty associated with this kind of work. It was sometimes expedient to combine experiments investigating different aspects of competition in bark, and hence to reduce the amount of work involved, by using the same controls. The NAN and non-aggressive isolates used throughout the work in this Chapter are listed in Table 9.1.

9.2.1 Competitive Establishment of Colonies From Mixed Spore Inocula

Two preliminary experiments were carried out in culture, looking at the influence of relative growth rate and the ratio of NAN to non-aggressive spore numbers on the establishment from mixed spore suspensions of NAN and non-aggressive isolates. These experiments identified a suitable range and intervals of incubation temperatures giving small but crtical differences in relative growth rate for particular combinations of isolates in equal ratio spore mixtures. They also indicated the sort of ratios of NAN to non-aggressive spores at which a slower growing isolate would be likely to be able to overcome its growth rate disadvantage. The following more detailed experiment was then undertaken.

Single isolate suspensions were prepared by incubating for 3 days in Tchernoffs medium on a box shaker. Four haemocytometer counts were made for each isolate to determine the number of spores per ml, and then all spore suspensions were adjusted to the same density by the addition of small volumes of sterile distilled water. The mixed isolate spore suspensions were prepared from the standardised single isolate suspensions. ESA plates were inoculated centrally with a 10 µl drop applied from a Finnpipette. All spore suspensions were agitated thoroughly on a rotary mixer before removing aliquots for haemocytometer counts, the preparation of mixed suspensions, and for the inoculation of plates.

To investigate the influence of relative growth rate the following equal ratio spore mixtures were prepared; two fully vegetatively incompatible NAN isolates of known penetrating ability (C112 and S144, Brasier, 1984 and unpublished data), two fully vegetatively

Table 9.1 NAN and Non Aggressive Isolates used for Investigation of Critical Aspects of Competition

Isolate	Description	Sampling date	Source*
NAN			
C112	B mating type	8.83	Chichester, CMB
S144	A mating type	8.83	Southampton, CMB
AL-D18	B mating type NAN supergroup	10.83	Alton wych elm, saprotrophic
HAY-51	A mating type	7.83	Haycocks, Mersea Island, saprotrophic phase
BH-T2	B mating type	7.84	Barrow Hill Farm, Mersea Island
BT-T16	B mating type	7.84	Barrow Hill Farm, Mersea Island
non-aggressive			
P82	B mating type	1980	Poland, CMB
H830	A mating type non-aggressive supergroup	1980	Vermont, USA, DRH
SS-A10	B mating type	9.84	Sancti Spiritus, Spain, saprotrophic phase
SS-A21	A mating type	9.84	Sancti Spiritus, Spain, saprotrophic phase
Es 70	A mating type	9.84	Casa de Campo, Madrid, CMB
Es 90	B mating type	9.84	Casa de Campo, Madrid, CMB

* Pathogenic phase isolates unless stated.
CMB. From the collection of C.M. Brasier.
DRH. Sampled by D.R. Houston.
All other isolates sampled by the author.

incompatible non-aggressive isolates (P82 and H830), and C112, S144, P82 and H830 in all four pairwise combinations of NAN and non-aggressive isolates. Four replicate plates were inoculated with each isolate and isolate combination. Five sets of plates were inoculated for incubation at 20, 22.5, 25, 27.5 and 30℃ .

To investigate the influence of the ratio of NAN to non-aggressive spores, the following spore concentration ratios of C112 with P82, and S144 with H830 were prepared for incubation at 20℃ , 1:10, 1:50, 1:100, and 1:200, and for incubation at 30℃ , 10:1, 20:1, 50:1 and 100:1. Four replicate plates were inoculated for each isolate combination and ratio. Since the work was carried out as a combined single experiment with that on the influence of relative growth, the single isolate and equal ratio inoculations in the first part also served as controls in the second.

Two diameters of each colony establishing from the spore drops were measured after 2 and 6 days incubation, and radial growth rate calculated in mm/day. All plates were removed from the incubator after 8 days, by which time the agar surface was covered, and incubated in diffuse daylight at ambient temperature for a further 5 days to encourage the development of synnemata.

The colonies establishing on each plate were then examined. In many cases where clearly only one isolate had established from the mixed isolate spore inoculum, it could be identified from its morphology by comparison with the morphology of colonies from the control single isolate spore inocula. A subculture was taken from the margin of each colony for confirmation. If from mixed NAN and non-aggressive spore inocula, the subcultures were identified to subgroup by their morphology after incubation on MEA at 25℃ in darkness for 3-4 days, followed by a further period in diffuse daylight at ambient temperature. Subcultures from within-subgroup control mixtures were identified in vc tests against the original isolates, in 4x4 patterns as described in Chapter 2.3.

Where both isolates had established, typically as distinct sectors, a diagram was made of each plate to estimate the area occupied by each subgroup. Subcultures were then made from each morphological type of sector and identified as described above. A similar approach was used in an experiment to examine relative establishment of NAN and non-aggressive equal ratio spore mixtures inoculated into elm bark. The inoculation method described in Chapter 2.9 was used with

logs of an English elm from Friston Forest, East Sussex. Standardised single isolate spore suspensions of four NAN isolates, C112, S144, AL-D18 and HAY-51, and four non-aggressive isolates, P82, H830, SS-A10, and SS-A21, were prepared as described above. The following equal ratio mixed isolate spore suspensions were then prepared: C112 with S144 (both NAN), P82 with H830 (both non-aggressive), C112 with P82, C112 with H830, S144 with P82, S144 with H830, AL-D18 with SS-A10, and HAY-51 with SS-A21. Ten replicate inoculations were made of each of the single isolate and mixed spore suspensions, and the whole experiment was duplicated for incubation at c.17℃ (range 15-19℃) and c.27.5℃ (range 25-30 ℃). Temperatures during the experiment were recorded using thermographs.

Destructive assesments were carried out after 11 weeks incubation at c.17℃ and 10 weeks at c.27.5℃ , using the method of Webber (1979). The bark around each inoculation point was carefully removed using a mallet and chisel,and a tracing made of the lesion at its maximum extent. Lesion areas were calculated from the weight of cut-out tracings.

The isolate establishing from a mixed inoculation was determined by re-isolation. A bark chip was taken from each side of the inoculation point and another from each end of the lesion. These were plated onto MEA+C+S and incubated at c.27℃ for 5-7 days. Isolations originating from mixed NAN and non-aggressive inocula were identified to subgroup by their morphology. Isolations from within-subgroup mixed inocula were identified from vc tests in 4x4 patterns (Chapter 2.3) against the original isolates.

In some cases entire lesions were cut out and incubated at c.27 ℃ on water agar containing cycloheximide and streptomycin at the usual concentrations. Stab isolations were made onto MEA+C+S from synnemata developing after several days incubation and identified as described above.

9.2.2 The Penetration Effect in VC Reactions between Opposed NAN and Non-Aggressive Isolates

Reactions between NAN and non-aggressive isolates opposed in culture were initially examined by pairing isolates on ESA and incubating at temperatures between 20 and 30℃ . The resulting reactions showed some characteristics of vc reactions within each subgroup as previously described (Brasier, 1984; Chapters 1.5 and 6).

The penetration effect was demonstrated by characterising synnematal stab isolations made from within colonies of isolates of both subgroups. However, because of the inconsistent production of synnemata by non-aggressive isolates this method did not allow detailed investigation of penetration.

Mycelial penetration was therefore measured between opposed carbendazim and iprodione tolerant isolates using the comb re-isolation method described in Chapter 2.8. Fungicide tolerant mutants of the respective NAN and non-aggressive isolates were developed using the methods described in Chapter 2.6 and 2.7.

Carbendazim and iprodione tolerant mutants of the NAN isolates C112 and S144, and the non-aggressive isolates P82 and H830, were opposed in all six pairwise combinations on ESA. In addition, a control compatible pairing was made of carbendazim tolerant C112 against iprodione tolerant C112. Two replicate pairings were made of each reciprocal isolate combination, as described in Chapter 2.8.

The work was repeated twice, each repeat experiment attempting to improve the basic method and extend the number of different incubation temperatures. The first two experiments were replicated for incubation at 20, 25 and 30°C , and the third for incubation at 20, 22.5, 25, 27.5 and 30°C . Relative penetration between NAN and non-aggressive isolates was also measured in elm bark as part of the experiment measuring penetration between NAN isolates (Chapter 5). The isolate combinations were the same as those used in agar culture (see above), plus a control self-compatible pairing of the non-aggressive isolate P82. Five replicate pairings of each reciprocal marker combination were made for each pair of isolates, and the experiment was duplicated for incubation at c.17 °C (range 15-19 °C) and c.27.5°C (range 25-30 °C). Temperatures were recorded using thermographs.

A wild-type, carbendazim tolerant and iprodione tolerant mutant of each isolate was also inoculated singly into bark, and its relative growth rate estimated from the size of lesion produced. Ten replicate inoculations were used for each isolate variant.

Destructive assessments were carried out after 13 weeks incubation. Penetration was measured by selective re-isolation onto MEA + carbendazim or iprodione, as described in Chapter 5.3. Lesion areas were again calculated from the weight of cut out tracings of exposed lesions.

9.2.3 Influence of Residual Host Resistance on Growth in Bark

The growth of NAN and non-aggressive isolates in untreated bark of a recently felled elm was compared to growth in the bark of logs from the same tree which had been killed with a 25 krad dose of gamma radiation. The radiation treatment was carried out by Isotron PLC of Swindon, Wiltshire.

The experiment was firstly undertaken using the standard inoculation method (Chapter 2.9). However, this was only a partial success, since it resulted in an unrealistically rapid growth rate in irradiated bark. This was probably due to the inoculation of some spores into previously intact xylem vessels, followed by rapid suction spread as the vessels embolized, and subsequent growth back out of the xylem into the bark. A second, more limited experiment was therefore carried out using the following modified inoculation method.

An approximately 10x10 mm square of outer bark was cut away using a scalpel, and a smaller square of inner bark of c.3x3 mm removed, taking care not to make contact with the xylem. Two drops of the spore suspension were inoculated into this hole, the inner and outer bark pieces replaced and the wound sealed with PVC tape.

The initial partially successful experiment involved six NAN isolates, C112, S144, AL-D18, HAY-51, BH-T2 and BH-T16, and six non-aggressive isolates, P82, H830, SS-A10, SS-A21, ES70 and ES90. Six replicates of each isolate were inoculated, except for C112 and P82 which had 12 replicates. Equal ratio spore mixtures of C112 with P82 and S144 with H830 were also inoculated with 12 replicates of each. Two sets of inoculations were made; the first into untreated bark and the second into irradiated bark. Both were incubated at c.27.5 C° (range 25-30 °C) , and a thermograph record kept of temperatures during the experiment.

Assessment of lesion area in healthy logs was made after 9 or 10 days from tracings of exposed lesions. The radiation treatment strongly discoloured the bark so that the lesions could not be seen. Assessment of growth in irradiated logs was therefore made by re-isolation. After 8 or 9 days incubation, the bark was carefully removed and thin strips of bark c.2 mm wide and totalling 100-150 mm in length, were cut along the grain in each direction from the inoculation point. The strips were plated out on MEA+C+S, incubated at c.27 °C for 2-3 days, and the furthest point along the strips at which

O.ulmi grew out recorded. The sum of the distances grown from the inoculation point in each direction was taken as the lesion length. Growth from strips from mixed subgroup inoculations was re-examined after further incubation to identify the subgroup or subgroups present. Establishment from mixed subgroup inoculations in untreated bark was assessed by re-isolation as described previously.

The second experiment used the same NAN and non-aggressive isolates, although only one equal ratio spore mixture, C112 with P82, was inoculated. In the irradiated logs six replicates were used for single isolates and 12 for the subgroup mixture. In the untreated logs 5 and 10 replicates were used respectively. Both sets of logs were incubated at c.27℃ (range 25-32℃), and a thermograph record kept of temperatures during the experiment.

The pattern of lesion development in untreated logs using the modified inoculation method made it difficult to obtain an accurate measurement of lesion area. Assessments of the maximum lesion length in each direction along the grain from the inoculation point were therefore made after 7 days incubation. Lesion lengths in the irradiated logs were assessed by the same procedure after 4 days incubation.

The short period of incubation and difficulties in maintaining an even temperature during summer necessitated a more accurate estimate of the mean temperature than had been made for previous experiments. To do this, the thermograph records were photocopied and cut along a line drawn at a known temperature through the trace. Parts of the trace above and below the line were cut out and weighed. Differences in weight of areas above and below the line were used to calculate any deviation from the temperature represented by the line, and therefore the mean temperature.

To provide a comparison of growth in bark with growth in culture, growth rate on MEA at 20℃ under standard conditions, and at c.27℃ under the same conditions as those used for the log experiments, was measured as described in Chapter 2.2. In the first experiment, two replicates of each isolate were used, but this was increased to four in the second experiment.

9.3 RESULTS

9.3.1 Competitive Establishment of Colonies from Mixed Spore Inocula

Data for the establishment of isolates on ESA from equal ratio mixtures of NAN and non-aggressive spores are summarised in Table 9.2, and the general appearance of the colonies illustrated in Plate 9.1. At temperatures below 25℃ only the NAN isolates established, but at 27.5 and 30 ℃ establishment depended on the particular combination of isolates. Of the NAN isolates, C112 was more successful than S144, and this same phenomenom also occurred in the results for establishment from a mixture of these two isolates. Of the non-aggressive isolates, P82 was more successful than H830, and this was again reflected in the results for competition betwen them. Thus, even when in competition at 30℃ with H830, C112 was still able to form some sectors to the edge of the plate; but in competition with P82 at 27.5℃ , S144 was unable to grow far beyond the inoculum.

The results can only be partially explained in terms of growth rate differences between the isolates when grown in single culture (Figure 9.1). The NAN isolates were faster than the non-aggressives at temperatures below 27.5℃ , but at 27.5℃ H830 had the fastest growth rate, S144 and P82 almost the same growth rate, and C112 the slowest growth rate. Predictions based entirely on intrinsic differences in growth rate would therefore suggest somewhat different results from those presented in Table 9.2.

However, the results are clarified if the growth rates of colonies developing from mixed inocula are taken into account (Figure 9.2). Considering the mixture of C112 and S144, the growth rate of colonies from mixed inocula was greater than that of either of the isolates alone. It seems that even though C112 was slower growing than S144 in single culture at temperatures above 22.5℃ , it was in some way able to increase its growth rate in the mixed culture, and so successfully outcompete S144. In contrast, the growth rate of the two non-aggressive isolates P82 and H830 in mixed culture was almost identical to that of two isolates grown alone.

A similar phenomenom to that found with the NAN isolates in mixed culture was seen in the growth rates of colonies developing from mixed NAN and non-aggressive inocula (Figures 9.1 and 9.2). Thus the establishment of NAN isolates in competition with non-aggressive isolates where consideration of growth rates in pure

Table 9.2 Comparative Isolate Establishment from Mixed NAN and Non-Aggressive Spore Inocula on ESA

Isolates	Isolate establishing from mixed spore inoculum *				
	Incubation temperature (°C)				
	20	22.5	25	27.5	30
Within subgroup					
C112 and S144 (NAN)	C112 dominating most of the colony. Felty area around inoculum where both isolates were assumed to be present. Felty area irregular in outline up to 25°C, sometimes reaching almost to margin. Decreasing in diameter from c.40 to 13mm with increasing temperature.				
P82 and H830 (non-aggressive)	Neither dominant. Some clearly defined sectors, but otherwise both isolates apparently growing together.			P82 dominating most of colony, but a felty area around the inoculum c. 15-20mm in diameter.	
NAN and non-aggressive					
C112 and P82	C112 only	C112 only	C112 only	C112 only	P82 only
C112 and H830	C112 only	C112 only	C112 only	C112 only	H830 dominating most of colony but some sectors of C112 reaching to margin.
S144 and P82	S144 only	S144 only	S144 only	P82 only	P82 only
S144 and H830	S144 only	S144 only	S144 only	H830 dominating most of colony. Some small sectors of S144 reaching to margin.	H830 only

* Four replicates incubated for 8 days at the specified temperature in darkness, followed by 5 days at ambient temperature in diffuse daylight

Plate 9.1 Establishment from Equal Ratio Spore Mixtures of NAN and Non-Aggressive Isolates at Different Incubation Temperatures

Top row, C112 (NAN) with H830 (non-aggressive), and bottom row S144 (NAN) with H830, from left to right incubated at 25, 27.5 and 30°C. At 25°C only the NAN isolates have established. At 27.5°C, H830 has failed to establish in competition with C112, but both H830 and S144 have established in competition with each other. At 30°C, both C112 and H830 have established, but S144 has failed to establish in competition with H830.

25 27.5 30°C

Figure 9.1 Radial Growth Rate of NAN and Non-Aggressive Isolates on ESA at Different Temperatures

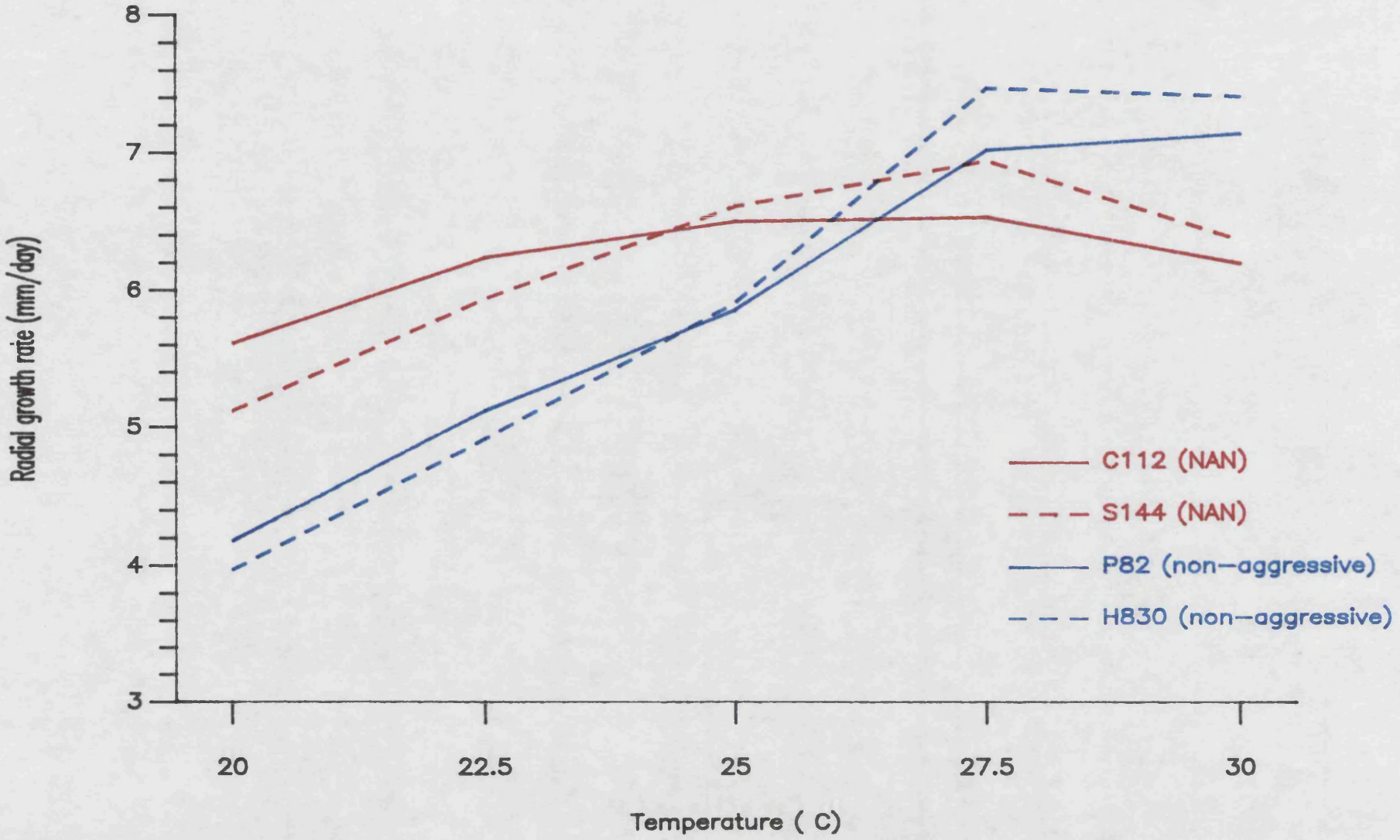
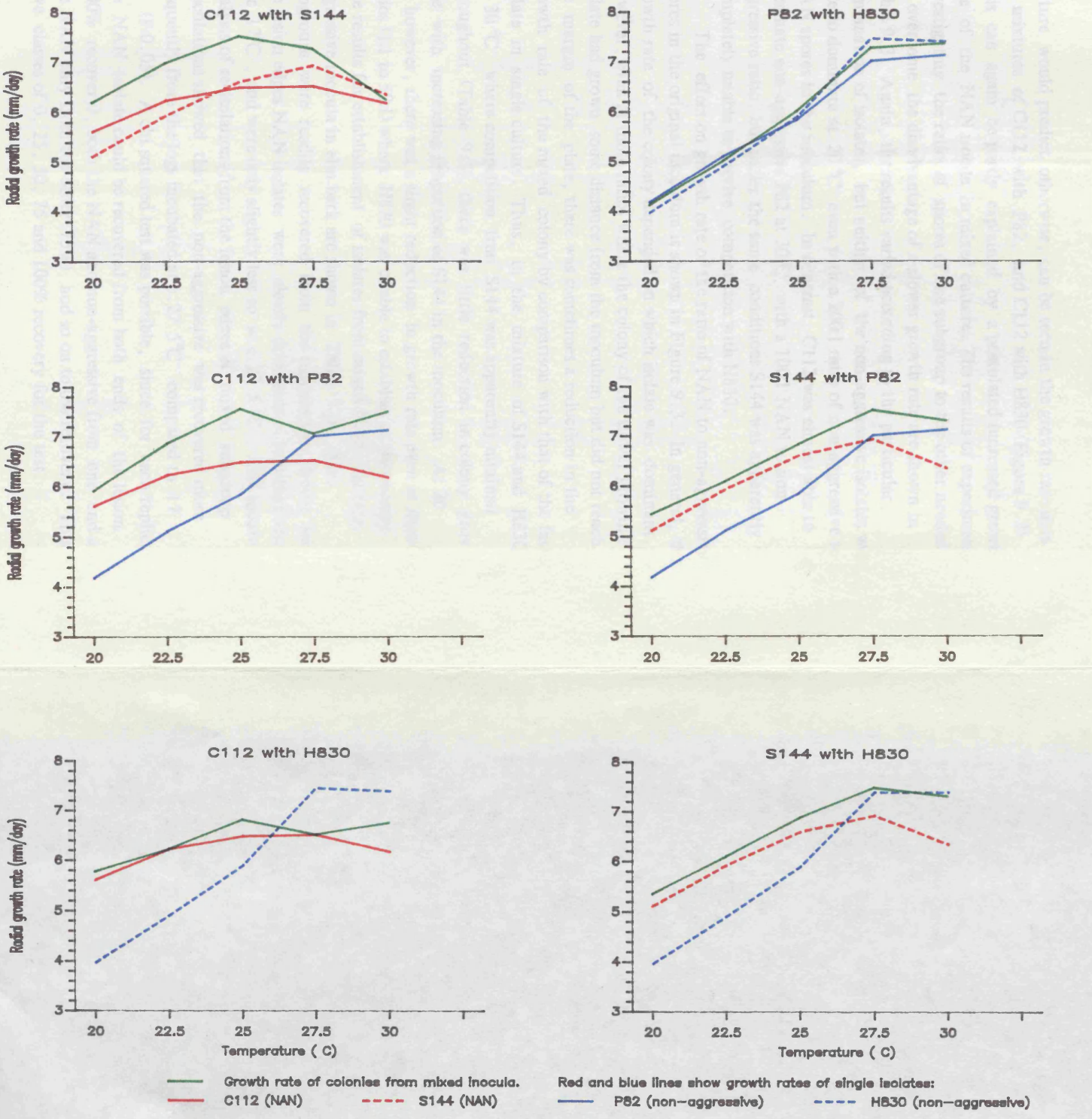


Figure 9.2 Radial Growth Rate of Colonies Establishing on ESA from Mixed NAN and Non–Aggressive Spore Inocula



culture would predict otherwise, can be seen in the growth rate data for mixtures of C112 with P82, and C112 with H830 (Figure 9.2). This can again be partly explained by a postulated increased growth rate of the NAN isolate in mixed culture. The results of experiments investigating the ratio of spores of one subgroup to the other needed to overcome the disadvantage of a slower growth rate are shown in Table 9.3. Again, the results varied according to the particular combination of isolates, but neither of the non-aggressive isolates were able to dominate at 20 ℃ even with a 200:1 ratio of non-aggressive to NAN spores in the inoculum. In contrast, C112 was almost able to dominate non-aggressive P82 at 30℃ with a 100:1 NAN to non-aggressive ratio, but under the same conditions S144 was apparently completely unable to survive competition with H830.

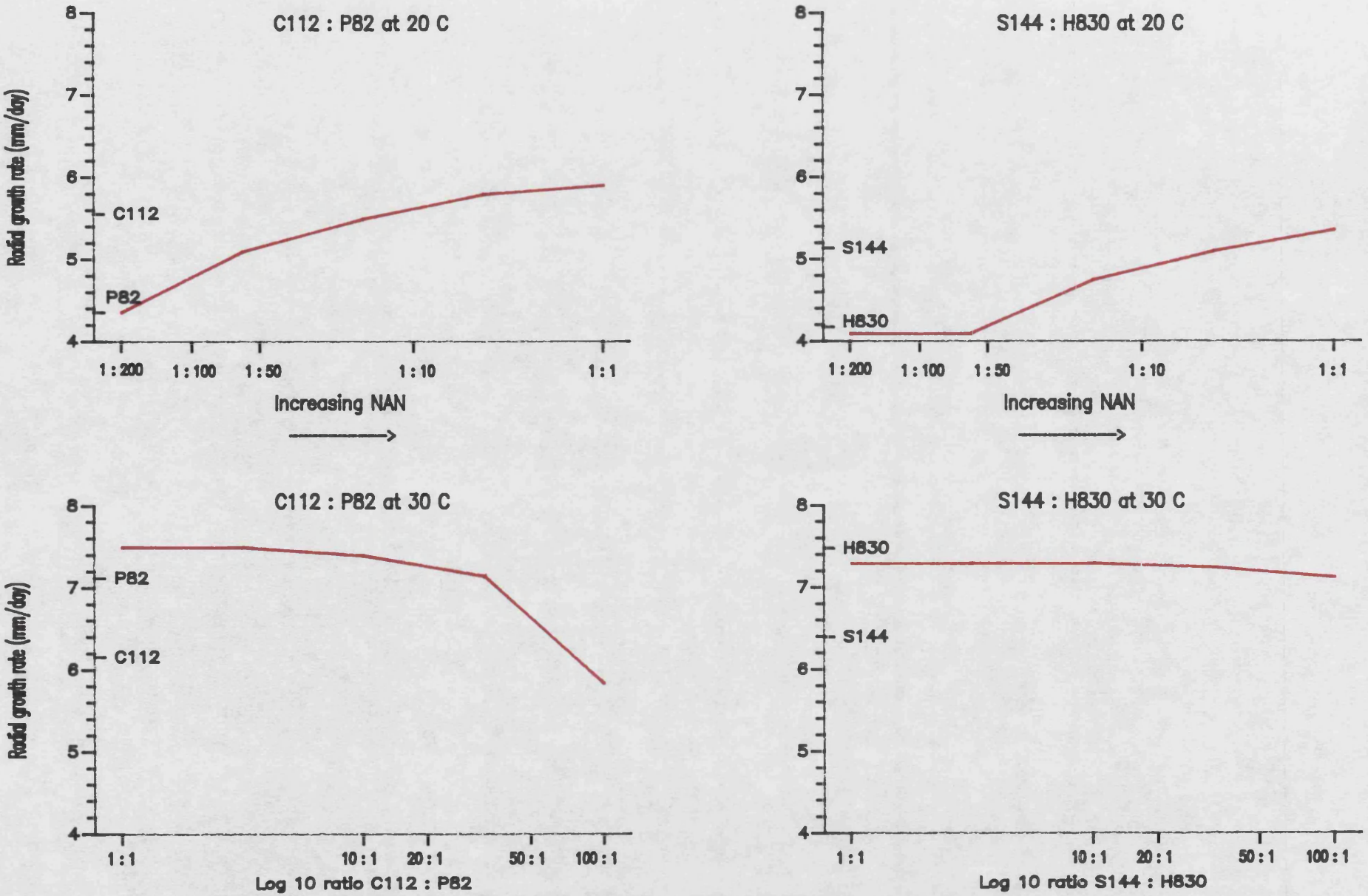
The effect on growth rate of the ratio of NAN to non-aggressive spores in the original inoculum is shown in Figure 9.3. In general, the growth rate of the colony depended on which isolate was dominant. However, even at those ratios where the colony of the slower growing isolate had grown some distance from the inoculum but did not reach the margin of the plate, there was sometimes a reduction in the growth rate of the mixed colony by comparison with that of the faster isolate in single culture. Thus, in the mixture of S144 and H830 at 30 ℃ where competition fron S144 was apparently minimal throughout (Table 9.3), there was little reduction in colony growth rate with increasing proportion of S144 in the inoculum. At 20 ℃ however, there was a slight reduction in growth rate even at those ratios (1:1 to 50:1) where H830 was unable to establish at the margin. The results for establishment of isolates from mixed NAN and non-aggressive inocula in elm bark are shown in Table 9.4. Both subgroups were readily recovered from the inoculation points, but at the lesion edges NAN isolates were clearly dominant in logs incubated at c.17℃ , and were only slightly less so at c.27.5 ℃ . Chi squared analysis of re-isolations from the lesion edges of mixed subgroup inoculations showed that the non-aggressive was recovered more frequently from the logs incubated at c.27.5℃ compared to 17 ℃ ($P<0.05$). A chi squared test was possible, since for each replicate the NAN isolate could be recovered from both ends of the lesion (100% recovery), both the NAN and non-aggressive from one end and the NAN only from the other (75%), and so on to 0% recovery. This gave classes of 0, 25, 50, 75 and 100% recovery for the test.

Table 9.3 Comparative Isolate Establishment on ESA from Mixed NAN and Non-Aggressive Spore Inocula of Varying Ratios

Isolates and incubation temperature	Isolates establishing from mixed spore inoculum*				
NAN and non-aggressive	Ratio of NAN to non-aggressive spores				
C112 and P82 at 20°C	1:1	1:10	1:50	1:100	1:200
	C112 dominating most of colony. Felty area around inoculum, increasing from c. 16 to 45mm in diameter with increasing proportion of P82.				Large sectors of both isolates, P82 occupying slightly larger area
C112 and P82 at 30°C	1:1	10:1	20:1	50:1	100:1
	P82 dominating most of colony. Felty area around inoculum, increasing from c. 20 to c. 55mm in diameter with increasing proportion of C112.				C112 dominating colony, except for one small sector of P82. Slightly felty area around inoculum c. 20mm in diameter.
S144 and H830 at 20°C	1:1	1:10	1:50	1:100	1:1200
	S144 dominating most of colony. Felty area around inoculum, increasing from c.20 to c.45mm in diameter with increasing proportion of H830			S144 dominating most of colony Some small sectors of H380	S144 dominating much colony. Some large sectors of H380
S144 and H380 at 30°C	1:1	10:1	20:1	50:1	100:1
	H830 dominating most of colony. Felty area around inoculum increasing from c. 16 to c. 23mm. in diameter with increasing proportion of S144.				

* Four replicates incubated for 8 days at specified temperature in darkness, followed by 5 days at ambient temperature in diffuse daylight.

Figure 9.3 Radial Growth Rate of Colonies Establishing on ESA from Mixed NAN and Non-Aggressive Spore Inocula of Varying Ratios



Growth rates of single cultures of each isolate shown on vertical axis.

Table 9.4 Establishment in Elm Bark from Mixed NAN and Non-Aggressive Spore Inocula

Isolates	% recovery of NAN or first named isolate*			
	From inoculation point		From lesion edge	
	17°C	27.5°C	17°C	27.5°C
Within subgroup				
C112 and S144 (NAN)	50	50	58	63
P82 and H830 (non-aggressive)	38	75	100	83
NAN and non-aggressive				
C112 and P82	65	65	98	79
C112 and H830	80	56	95	100
S144 and P82	45	55	98	50
S144 and H830	62	48	98	80
AL-D18 and SS-A10	82	56	100	100
HAY-51 and SS-A21	56	37	82	78

* Isolations made from 10 replicates after 11 and 10 weeks incubation at c.17 and c.27.5°C respectively, as described in text. Percent recovery calculated only from those bark chips giving O.ulmi. Chips giving both NAN and non-aggressive isolates were counted as half scores for each subgroup.

The results can be most simply explained by the differences in areas of lesions (as a measure of growth rate) produced by the NAN and non-aggressive isolates alone in elm bark (Table 9.5). The NAN produced larger lesions than the non-aggressive isolates at both c.17 and c.27.5℃ ($P<0.001$), although the difference was not as great at the higher temperature. At 27.5℃ growth may have stopped before the assessments were made, since much of the bark was dry by the end of the test. This may have reduced the overall sensitivity of the experiment.

The size of lesions formed by mixed subgroup inoculations was not significantly different from that of NAN isolates alone. However, the mean lesion areas of five out of the six mixed subgroup inoculations at c.17 ℃ were greater than the lesion areas produced from the respective NAN isolates alone (Table 9.5). It is therefore likely that when in competition with non-aggressive isolates growth rate of NAN isolates was increased, but not enough to be detected by this experimental procedure.

9.3.2 The Penetration Effect in VC Reactions between Opposed NAN and Non-Aggressive Isolates

The results of the second and third experiments investigating the effects of temperature and relative growth rate on mycelial penetration in culture between fungicide tolerant NAN and non-aggressive isolates are summarised in Tables 9.6 and 9.7 respectively. Results of the first preliminary experiment have not been shown. Incubation temperature, and by inference relative growth rate, was shown to influence the depth of penetration.

NAN isolates penetrated non-aggressive isolates to a fairly consistent depth up to 25 or 27.5℃, depending on the particular combination of isolates. At 30 ℃ there was no effective penetration by the NAN isolates, based on the results of controls. Non-aggressive isolates penetrated NAN isolates to a surprising extent even at 20℃, and showed some increase in the depth of penetration with increasing temperature.

The results of the two experiments conflict with respect to the the relative depth of penetration by the two subgroups, especially at 20 ℃. The second experiment suggested that at 20℃ NAN isolates penetrated non-aggressive isolates more than they were themselves penetrated, but in the third experiment penetration was more equal. This could be due to differences in the length of incubation of the two

Table 9.5 Growth in Elm Bark from Single Isolate and Mixed NAN and Non-Aggressive Spore Inocula

Isolates	Mean lesion area (sq cm)* Incubated at c.17°C		Incubated at c.27.5°C	
NAN isolates				
C112	3.4 ± 1.16}		3.6 ± 2.56}	
S144	2.8 ± 1.21}		2.6 ± 1.15}	
AL-D18	4.2 ± 2.76}	3.2 ± 1.79	4.6 ± 2.64}	3.4 ± 2.09
HAY-51	2.5 ± 1.28}		2.7 ± 0.99}	
C112 and S144	4.0 ± 2.26		2.9 ± 1.50	
non-aggressive isolates				
P82	1.8 ± 0.81}		2.7 ± 1.26}	
H830	2.1 ± 0.95}		2.8 ± 1.11}	
SS-A10	1.2 ± 0.45}	1.7 ± 0.75	1.6 ± 0.76}	2.4 ± 1.19
SS-A21	1.8 ± 0.49}		2.6 ± 1.34}	
P82 and H830	2.2 ± 1.15		3.0 ± 1.49	
NAN and non-aggressive isolates				
C112 and P82	4.4 ± 3.51}		2.6 ± 1.29}	
C112 and H830	3.9 ± 1.60}		2.8 ± 1.07}	
S144 and P82	4.0 ± 2.29}		2.6 ± 1.45}	
S144 and H830	3.5 ± 1.76}	3.8 ±2.26	3.4 ± 1.78}	3.0 ± 1.43
AL-D18 and SS-A10	3.9 ± 2.76}		3.4 ± 1.72}	
HAY-51 and SS-A21	2.9 ± 1.46}		3.0 ± 1.32}	

* Means of 10 replicates with a total of three missing values. Assessed after 11 and 10 weeks incubation at c.17°C and c.27.5°C respectively.

ANOVA showed that NAN isolates produced significantly larger lesions than the non-aggressive isolates at both incubation temperatures; and that the non-aggressive itself produced significantly larger lesions at 27.5 than at 17°C ($P < 0.01$). Standard error, 1.68.

Table 9.6 Relative Mycelial Penetration between Marked NAN and Non-Aggressive Isolates Opposed on ESA - Second Experiment

Isolates	Relative mycelial penetration of each isolate (mm)*		
	20°C	25°C	30°C
Within subgroups			
C112 v. C112 (NAN)	2,3	2,2	3,3
C112 v. S144 (NAN)	17,17	13,13	15,6
P82 v. H830	6,6	14,17	9,19
NAN v. non-aggressive			
C112 v. P82	11,18	14,16	25,2
C112 v. H830	15,17	12,20	15,2
S144 v. P82	13,24	17,19	27,1
S144 v. H830	12,20	16,20	12,8
mean NAN v. non-aggressive	13,20	15,19	10,3

* Measured by selective re-isolation as described in text.
First figure in each pair is penetration of the first named isolate by the second, and similarly the second figure is penetration of the second named isolate by the first.
Means of two replicates of each reciprocal marker combination, assessed after 27 days incubation.

Table 9.7 Relative Mycelial Penetration between Marked NAN and Non-Aggressive Isolates Opposed on ESA - Third Experiment

Isolates	Relative mycelial penetration of each isolate (mm)*				
	20°C	22.5°C	25°C	27.5°C	30°C
Within subgroup					
C112 v. C112 (NAN)	3,3	2,3	4,3	1,3	1,2
C112 v. S144 (NAN)	16,11	13,13	12,10	14,9	14,4
P82 v. H830 (non-aggressive)	11,6	15,7	17,9	15,12	17,11
NAN v. non-aggressive					
C112 v. P82	11,11	13,8	10,7	12,8	19,2
C112 v. H830	12,8	12,8	10,10	17,8	17,3
S144 v. P82	9,16	12,14	12,15	14,7	11,3
S144 v. H830	13,15	11,14	17,18	16,16	9,8
mean NAN v. non-aggressive	11,13	12,11	12,13	15,12	14,4

* Measured by selective re-isolation as described in text.
First figure in each pair is penetration of the first named isolate by the second, and similarly the second figure is penetration of the second named isolate by the first.
Means of two isolates of each reciprocal marker combination, assessed after 21 days incubation.

experiments.

Although the experiments were carried out with carbendazim and iprodione tolerant mutants, growth rates at temperatures from 20-30 °C can be compared for their sensitive or wild-type counterparts on ESA (Figure 9.1). Although carbendazim tolerant mutants are slightly slower growing than wild-types (Webber, 1983), while iprodione tolerant isolates are faster (Mitchell, 1987), these differences would probably more or less cancel each other out. The results in Tables 9.6 and 9.7 are based on means of reciprocal combinations of the two markers, and no differences were detected in the penetration of iprodione tolerant isolates by carbendazim tolerant isolates, or *vice versa*.

The actual and relative depth of penetration in within subgroup pairings of the NAN and non-aggressive isolates (Tables 9.6 and 9.7), showed some variation with temperature. Again there were differences between the two experiments. However, the differences in depth of penetration at 20°C compared to 30 °C were small compared with the fairly large differences in growth rates of the isolates at these temperatures (Table 9.10). The results of the investigation of the role of the penetration effect in elm bark are summarised in Table 9.8. Considerable variation was found in the size of lesions produced, particularly by the iprodione tolerant isolates (Table 9.9), and this reduced the usefulness of the experiment. Similar difficulties were described in Chapter 5 regarding the investigation of penetration between NAN isolates opposed in elm bark, which was carried out as part of the present experiment.

However, by combining the data for the two incubation temperatures it was possible to demonstrate that there was a significantly greater overlap of lesions of NAN and non-aggressive isolates as compared to control compatible pairings of the same isolate ($P<0.05$). This can be interpreted as the first demonstration of the penetration effect between NAN and non-aggressive isolates in elm bark.

The results of the pairings between the non-aggressive isolates P82 and H830 did not show any greater overlap than the control self-compatible pairings. This was probably due to the generally small size of lesions produced by non-aggressive isolates, rather than the absence of the penetration effect, since the effect was demonstrated between these two isolates in culture.

Table 9.8 Mycelial Penetration between Marked NAN and Non-Aggressive Isolates Opposed in Elm Bark

Isolates	Mean overlap between lesions (mm)*	
	Incubated at c.17°C	Incubated at c.27°C
Within subgroup		
C112 v. C112 (NAN)	1.4 ± 1.37	4.1 ± 4.93
P82 v. P82 (non-aggressive)	3.0 ± 1.70	0.6 ± 1.34
C112 v. S144 (NAN)	11.7 ± 3.05	9.6 ± 8.72
P82 v. H830 (non-aggressive)	2.3 ± 1.28	0
NAN v. non-aggressive		
C112 v. P82	1.9 ± 2.66	4.9 ± 5.11
C112 v. H830	5.5 ± 5.96	6.8 ± 7.12
S144 v. P82	5.6 ± 5.27	6.0 ± 7.32
S144 v. H830	3.6 ± 2.65	5.9 ± 4.76
mean NAN v. non-aggressive	4.2 ± 4.45	5.8 ± 5.85

* Measured by selective re-isolation after 13 weeks incubation, as described in text. Means of 5-9 replicates, combined data for reciprocal combinations of carbendazin and iprodrone fungicide tolerant markers.

ANOVA showed significantly less overlap in control self compatible pairings of the same isolate than in pairings of NAN and non-aggressive isolates, combining data for the two incubation temperatures ($P < 0.05$). Standard error, 4.54.

Table 9.9 Relative Growth of Wild-Types and Carbendazim and Iprodione Tolerant Mutants in Elm Bark

Isolate	Marker	Mean lesion area (sq cm)* Incubated at c.17°C	Incubated at c.27°C
C112	Wild-type	6.1 ± 4.32	4.2 ± 2.96
	Carbendazim tolerant	5.2 ± 6.49	3.4 ± 2.22
	Iprodione tolerant	2.8 ± 1.20	2.0 ± 0.67
S144	Wild-type	5.3 ± 2.90	5.3 ± 3.12
	Carbendazim tolerant	6.7 ± 4.17	3.9 ± 3.62
	Iprodione tolerant	2.0 ± 0.84	1.5 ± 0.57
P82	Wild-type	3.4 ± 3.41	2.9 ± 1.45
	Carbendazim tolerant	2.0 ± 1.85	2.6 ± 1.46
	Iprodione tolerant	1.3 ± 0.38	1.6 ± 1.06
H830	Wild-type	2.7 ± 2.56	1.9 ± 0.54
	Carbendazim tolerant	2.7 ± 1.49	2.3 ± 1.17
	Iprodione tolerant	1.2 ± 0.32	1.3 ± 0.60

* Mean of 10 replicates, with a single missing value. The experiment was assessed after 13 weeks incubation.

ANOVA showed that NAN isolates produced significantly larger lesions than non-aggressive isolates ($P < 0.001$); and that wild-types and carbendazim tolerant mutants produced significantly larger lesions than iprodione tolerant mutants ($P < 0.001$). Standard error, 2.53.

Assessment of penetration on the basis of characterization of synnematal stabs following damp chamber incubation generally supported the data obtained via the bark strip isolations. However, the number of replicates assessed in the former was limited by time, and this together with the poor production of synnemata by iprodione tolerant isolates, prevented a detailed comparison.

9.3.3 Influence of Residual Host Resistance on Growth in Bark

The sizes of lesions formed by NAN and non-aggressive isolates in untreated and irradiated bark at c.27℃ were converted to lesion extension rates. The results are given in Table 9.10. Also shown for comparison are their radial growth rates on MEA at 20 and 27.5℃ .

An analysis of variance showed as expected that on MEA at 20 ℃ NAN isolates were significantly faster growing than non-aggressive isolates ($P<0.001$), but there was no difference between them at 27.5 ℃ . There were no differences in the growth rate of NAN isolates on MEA at 20 ℃ compared to 27.5 ℃ , but non-aggressive isolates grew significantly faster at the higher temperature ($P<0.001$).

It is well established from previous work (Webber, 1979; Webber & Hedger, 1986), that NAN isolates produce larger lesions than non-aggressive isolates in elm bark at 15-20 ℃ . The first experiment (Table 9.10) showed that NAN isolates also produce significantly larger lesions than non-aggressive isolates at 27.5 ℃ ($P<0.001$), whereas on MEA or ESA (Figure 9.1) the growth rates of the two subgroups are typically more or less equal at this temperature (Brasier *et al.*, 1981). Although the data from the second experiment for lesion extension rates in untreated bark (Table 9.10) did not show any significant difference, this was probably due to the very short duration of the experiment. Evidence for the faster growth rate of NAN isolates in bark at c.27.5 ℃ is also given in Tables 9.5 and 9.9.

However, in irradiated bark at c.27.5℃ there was no difference between the lesion extension rates of the NAN and non-aggressive isolates. This supports the suggestion (Webber, 1979) that the faster growth rate of NAN isolates in untreated bark is due to their greater ability to overcome the residual host resistance of the elm bark. Interestingly, the lesion extension rates of the isolates in irradiated bark at c.27.5℃ were nearly twice their radial growth rates on MEA at the same temperature (Table 9.10), but were similar to their growth

Table 9.10 Relative Growth of NAN and Non-Aggressive Isolates on MEA and in Untreated and Irradiated Elm Bark

Isolates	Growth rate on MEA (mm/day)* 20°C	Growth rate on MEA (mm/day)* c.27.5°C	Lesion area (cm sq) Untreated bark+ c.27°C	Lesion extension rate (mm/day) Untreated bark# c.27.5°C	Lesion extension rate (mm/day) Irradiated bark x c.28°C
NAN					
C112	3.3 ± 0.16	2.8 ± 0.25	1.7 ± 0.40	1.5 ± 0.57	6.7 ± 1.10
S144	3.5 ± 0.21	3.3 ± 0.23	1.6 ± 0.28	1.2 ± 0.64	6.2 ± 1.35
AL-D18	3.5 ± 0.04	3.4 ± 0.48	1.8 ± 0.66	1.3 ± 0.94	5.8 ± 0.68
HAY-51	3.3 ± 0.20	3.1 ± 0.25	1.3 ± 0.27	1.3 ± 0.53	6.0 ± 1.36
BHF-2	3.9 ± 0.13	3.9 ± 0.32	1.3 ± 0.52	1.6 ± 1.17	5.0 ± 1.96
BHF-16	3.7 ± 0.19	3.3 ± 0.31	2.1 ± 0.58	2.0 ± 1.06	5.6 ± 0.40
mean NAN	3.5 ± 0.28	3.3 ± 0.44	1.7 ± 0.49	1.5 ± 0.79	5.9 ± 1.29
non-aggressive					
P82	3.0 ± 0.05	3.8 ± 0.08	0.9 ± 0.21	1.1 ± 0.58	6.3 ± 1.19
H830	2.3 ± 0.03	3.5 ± 0.10	1.2 ± 0.29	1.2 ± 0.67	5.9 ± 0.30
SS-A10	2.5 ± 0.18	3.4 ± 0.63	1.1 ± 0.28	1.5 ± 0.53	6.3 ± 0.68
SS-A21	3.0 ± 0.22	4.1 ± 0.19	1.2 ± 0.29	1.2 ± 0.53	6.1 ± 0.82
ES-70	2.9 ± 0.09	3.5 ± 0.10	1.1 ± 0.12	0.8 ± 0.47	5.6 ± 0.98
ES-90	2.7 ± 0.28	3.5 ± 0.13	1.3 ± 0.47	1.3 ± 0.59	6.2 ± 0.40
mean non-aggressive	2.7 ± 0.30	3.6 ± 0.35	1.1 ± 0.30	1.2 ± 0.54	6.1 ± 0.76

Table 9.10 continued

* Means of 4 replicates measured over a 5 day period.

+ From first experiment. Means of 12 replicates for C112 and P82 and means of 6 replicates for other isolates, measured after 9-10 days. One missing value.

From second experiment. Means of 4-6 replicates, measured after 7 days.

x From second experiment. Means of 4-5 replicates, measured after 4 days.

Each set of data was tested by ANOVA. For growth rate on MEA, NAN isolates were significantly faster than non-aggressive isolates at 20°C ($P<0.001$), but not at 27.5°C. The lesion areas of NAN isolates in untreated bark at 27.5°C were significantly greater than those of non-aggressive isolates ($P<0.001$). The lesion extension rates of NAN and non-aggressive isolates in irradiated bark at 27.5°C were not significantly different.

rates on ESA (Figure 9.1).

Non-aggressive isolates inoculated in mixtures with NAN isolates were recovered at higher frequencies (from either the lesion edges or from extreme sampling points) in irradiated as compared to untreated elm bark (Table 9.11). From the evidence of the preceeding experiments this is probably due to their having a more equal growth rate to the aggressive in irradiated bark.

Table 9.11 Establishment from Mixed NAN and Non-Aggressive Spore Inocula in Untreated and Irradiated Elm Bark at c.27.5°C

Isolates NAN and non-aggressive	% Recovery of NAN at lesion edge or extreme sampling point* Untreated bark			Irradiated bark	
	From Table 9.4+	First experiment#	Second experiment#	First experiment#	Second experiment#
C112 and P82	79	82	58	48	44
S144 and H830	80	85	-	73	-

* Isolations made as described in text. Percent recovery calculated only from those bark chips or strips giving O.ulmi. Chips or strips giving both NAN and non-aggressive isolates were counted as half scores for each subgroup.

+ Means of 10 replicates.

Means of 12 replicates.

9.4 DISCUSSION

The results illustrated the importance of growth rate in various aspects of competition between the NAN and non-aggressive subgroups. In culture, the relative growth rates of the NAN versus the non-aggressive isolates were determined by temperature and their intrinsic growth abilities. By contrast, in elm bark residual host resistance and the pathogenic capability to overcome it were shown to be of overriding importance. However, the results cannot be entirely explained on the basis of differences in growth rate, and the experiments revealed other aspects of the competitive abilities of the two subgroups.

When establishing on ESA from a mixed spore inoculum, NAN isolates seemed to have an advantage beyond that which would be predicted by their faster relative growth rate in pure culture (Table 9.2, Figure 9.1). The growth rates of colonies establishing from mixed inocula suggested that one of the NAN isolates at least, C112, was able to put on a spurt of growth rate (Figure 9.2). The non-aggressive isolates did not show this ability, which could also be important in competition between isolates of the same subgroup. A high ratio of spores of the less competitive isolate, whether NAN or non-aggressive, to that of the more competitive isolate was required to overcome this disadvantage (Table 9.3).

In natural (as opposed to irradiated) elm bark the outcome of competition was apparently determined by relative growth rate, but although non-aggressive isolates were more successful at c. 27 ℃ compared to c. 17℃ they still competed poorly (Tables 9.4 and 9.5). This can be best explained by the greater ability of NAN isolates to overcome the residual host resistance of the bark (Table 9.10). The outcome of competition between NAN and non-aggressive isolates was much more equal in irradiated elm bark without any host resistance, when incubated at a temperature giving more or less equal growth rates in culture (Table 9.11).

Thus, in natural situations when establishing in competition with each other in beetle galleries in dying elm bark, it can be predicted that the NAN subgroup will be at a strong advantage over the non-aggressive subgroup, regardless of temperature. Even if the ability to overcome host resistance was not involved, the non-aggressive subgroup would only be at an advantage at temperatures above c. 27℃ .

The considerable variation in the proportion of NAN and non-aggressive spores on beetles emerging from bark containing isolates

of both subgroups (Figures 8.2 and 8.4), suggests that the growth rate disadvantage of non-aggressive isolates when establishing in bark could sometimes be overcome by weight of numbers. Considered with the influence of residual host resistance, the data presented in Table 9.3 indicate that the natural balance in favour of the non-aggressive would have to be very high.

Some aspects of pathogenicity and host resistance are also likely to be involved in competition in feeding grooves. If so, the non-aggressive subgroup will be at a disadvantage and less likely to enter the xylem and infect the tree when competing with the NAN subgroup.

Residual host resistance will no longer play a part in competition once the bark is dead and completely colonised. The demonstration in 'natural' elm bark of the penetration effect between the two subgroups (Table 9.8) allows more detailed consideration of its likely importance during the later stages of the saprotrophic phase. The experiments in culture showed that although growth rate has some influence on the relative and actual depths of penetration between NAN and non-aggressive isolates, other factors are involved (Tables 9.6 and 9.7, Figure 9.1). These factors may be considered as intrinsic penetrating ability, and the heirarchical nature of the penetration effect among NAN isolates independent of growth rate (Brasier, 1984; 1986a) would have predicted their involvement.

The investigation of mycelial penetration in culture showed a surprising degree of penetration of NAN isolates by non-aggressive isolates, even at 20 ℃ . In the isolate combinations studied, below 30 ℃ the NAN isolates had either a slight advantage or the two subgroups were almost evenly matched (Tables 9.6 and 9.7). Only at 30 ℃ were the non-aggressive isolates clearly at an advantage. However, it should be remembered that the technique measured mycelial penetration by the selective re-isolation of fungicide tolerant mutants. Also, the enhanced sporulation, which is a feature of the penetration effect *in vitro*, was not assessed in elm bark, and may be at least as important as territorial invasion. In culture, the non-aggressive produces conspicuously fewer synnemata, and indeed this was the reason for needing to use fungicide tolerant isolates for investigation of the penetration effect.

SECTION III - CONCLUSIONS

The experiments attempting to measure competition between the aggressive and non-aggressive subgroups in mixed saprotrophic phase populations illustrated the considerable complexity of the relationship between host, pathogen and vector. Post-peak emerging beetles were found to carry a greater number of spores and a greater proportion of genotypes originating from the pathogenic phase. It is suggested that this is because later emerging beetles are from pupal chambers formed in the inner bark in contact with the xylem. Such pupal chambers are more likely to colonised by pathogenic phase isolates, and to contain synnemata or perithecia.

The quantity and quality of *O.ulmi* inoculum carried by emerging beetles is therefore likely to depend on the positions of their pupal chambers. The relative numbers of pupal chambers in the inner and outer bark varied between experiments in different years, but the factors influencing the positions of pupal chambers are largely unknown.

No differences were detected in the abilities of NAN and non-aggressive isolates to feed back from the pathogenic phase to the saprotrophic phase and to contribute to the sporeloads of beetles. Neither were differences detected in their abilities to contribute to sporeloads when originating from spores brought in by the previous generation of beetles. However, conditions for the two parts of the investigation were not identical.

The presence of a small proportion of NAN spores on the beetles used to carry non-aggressive spores into the bark of Trees 1, 2 and 3 gave an important insight into the interaction between the subgroups. Namely, that a low level of NAN spores on the beetles together with NAN isolates in the xylem appeared to greatly enhance the levels of NAN colonising pupal chambers and hence that carried by the next generation of beetles. The success of both wild-type NAN originating on the parent beetles, NAN *tol* from the pathogenic phase in the xylem, and recombinant progeny was increased. This apparent synergism could be a major factor in the replacement of the non-aggressive subgroup. Relative growth rate was shown to be critical to the outcome of colonisation from mixed NAN and non-aggressive spore inocula. In culture, relative growth rate was largely determined by temperature. However, when competing with other isolates the two NAN isolates used both increased their competitive success with a spurt of growth rate above that of their growth rate in single culture. The two non-

aggressive isolates used did not show this ability.

In bark, the ability to overcome the residual host resistance of dying bark was an overriding factor in determining relative growth rates of competing subgroups. This would undoubtedly give the NAN subgroup a decisive advantage over the non-aggressive subgroup when colonising bark from a mixture of spores carried into bark by breeding beetles.

In culture, the disadvantages of slower growth rate could be overcome by higher spore ratios of the slower isolate. However, the effect of residual host resistance would require very high ratios of non-aggressive to NAN spores for the former to colonise and compete from such spore mixtures in bark.

Relative growth rate also influenced the depth of mycelial penetration in pairings between NAN and non-aggressive isolates in culture. The experiments suggested that mycelial penetration of non-aggressive isolates by NAN isolates was probably greater than the penetration of NAN isolates by non-aggressive isolates. In bark, mycelial penetration was demonstrated between fungicide tolerant NAN and non-aggressive isolates. This enabled information from experiments in culture to be used for consideration of mycelial interactions taking place during the saprotrophic phase. During the early part of the saprotrophic phase the extent of penetration between isolates of the two subgroups will be influenced by growth rate advantage or disadvantage conferred by the ability to overcome residual host resistance. At later stages when the bark is dead, the effect of temperature on growth rate may be more important.

10 CONCLUDING DISCUSSION

Investigation of the saprotrophic phase of *O.ulmi* at sites with pure populations of either the NAN or non-aggressive subgroups (Sections I and II) revealed basic similarities in their population structures. The existence of a mosaic of different genotypes in the bark first described by Lea (1977) was both confirmed here for the NAN aggressive and demonstrated for the non-aggressive by using vegetative compatibility as a marker. It is suggested that the vc system functions to maintain and regulate the mosaic.

Detailed investigation of the NAN aggressive provided further strong evidence for a dynamic, highly competitive saprotrophic phase, as described by Brasier (1984, 1986a), Webber & Brasier (1984) and Webber *et al.*, (1987). The occurrence of a mosaic of genotypes means that there is intense intraspecific competition throughout the saprotrophic phase, from the initial establishment in beetle breeding galleries to the final colonisation of and sporulation in pupal chambers.

The results presented in Chapter 3 showed that several genotypes are likely to establish in each breeding gallery. They may be brought in by the male and female beetle, or also derive from the pathogenic phase in the underlying xylem. Competition between different genotypes could then result in mutual invasion of territory *via* the penetration effect, and the establisment of novel recombinant genotypes *via* mating. The penetration effect may also play a critical role in the colonisation of pupal chambers, where a stronger penetrating isolate may be stimulated to produce more synnemata, and so increase its chances of contributing to the sporeloads of emerging beetles.

The various interactions leading to the establishment of a highly diverse saprotrophic phase population will help to maintain a diverse pathogenic phase population. In Chapter 8, isolation experiments showed that most pupal chambers were colonised by more than one genotype, and in consequence most beetles carried spores of more than one genotype. This would be likely to lead to a genetically diverse pool of isolates causing infection in the xylem of healthy trees *via* beetle feeding grooves. Similarly, the next saprotrophic phase would be initiated by a genetically diverse population. Other factors, such as host selection, will also be acting on the *O.ulmi* population, and these may vary in their effect on epidemic front and post-epidemic populations.

Although competition during the saprotrophic phase will maintain diversity, it may also facilitate changes in the population in response to selection pressures. For example, the frequency of the NAN vc supergroup *w* allele was found to change in the Barrow Hill Farm population (Chapter 4) over the period 1983-85. The occurrence of a vc supergroup in the North American pathogenic phase sample of the non-aggressive subgroup (Chapter 7) suggested that there may be a similar response in both the aggressive and non-aggressive subgroups to episodic selection pressures at epidemic fronts. The evidence for the occurrence of intense competition within saprotrophic phase populations of either the NAN or non-aggressive subgroups can be extended to competition between them. The experiments investigating competition (Chapters 8 and 9) identified several important elements of this competition, and will be used to propose a model for the events leading to replacement of the non-aggressive by the aggressive. It is likely that these factors also influence competition between genotypes within a subgroup, although the outcome would probably not be as extreme. The proposed model, summarised in Figure 10.1, is as follows:

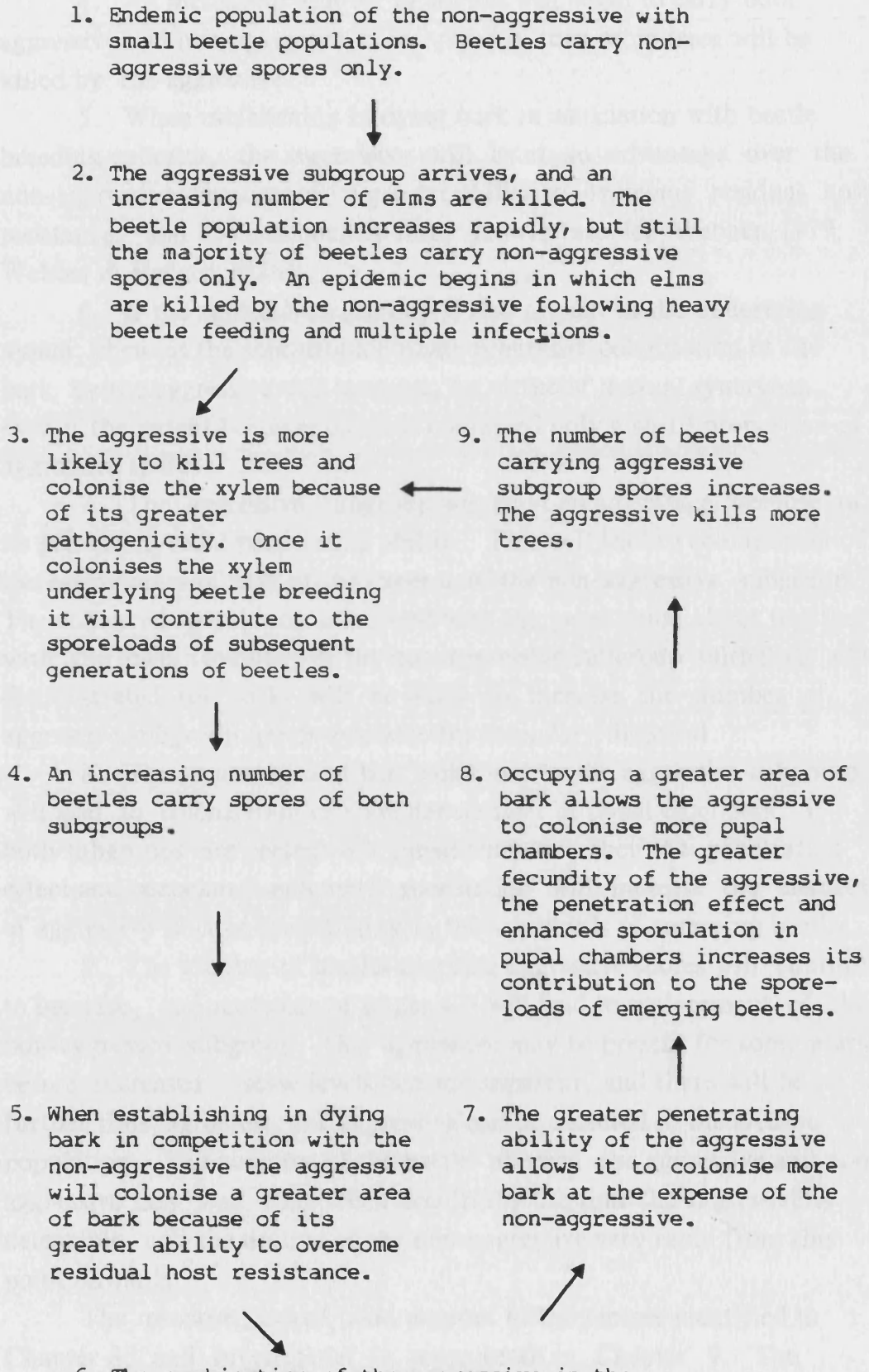
1. In 'pure' non-aggressive subgroup populations all beetles will carry only non-aggressive spores, related beetle populations will tend to be small and disease levels low.

2. Following the arrival of the aggressive subgroup, the breeding material available to beetles will increase. Consequently, beetle populations will increase, but initially the great majority of beetles will carry non-aggressive spores only. This will greatly increase the inoculum potential of the non-aggressive. An epidemic will be initiated, in which trees will be killed by the non-aggressive following heavy beetle feeding and multiple non-aggressive infections. Although the aggressive may at this stage be virtually undetectable in the *O. ulmi* population, replacement of the non-aggressive will begin.

This situation was predicted by Brasier (1983a), and has since been confirmed in studies of the early stages of epidemics initiated by the NAN aggressive in Spain and Portugal (Brasier, 1988).

3. Although the aggressive will be present at low levels, because of its greater pathogenicity it will be more likely to kill trees and colonise the xylem than the non-aggressive. Once it occupies large volumes of xylem it will be able to contribute to the sporeloads of the subsequent generation of beetles colonising the surrounding bark. Its

Figure 10.1 A Qualitative Model for the Replacement of the Non-Aggressive Subgroup



cytoplasmic fitness may also be enhanced by constant passage through the pathogenic phase (Brasier, 1986a; Rogers *et al.*, 1986a).

4. An increasing number of beetles will begin to carry both aggressive and non-aggressive spores, and in turn more trees will be killed by the aggressive.

5. When establishing in dying bark in association with beetle breeding galleries, the aggressive will be at an advantage over the non-aggressive because of its greater ability to overcome residual host resistance, and its consequently faster growth rate (cf Webber, 1979; Webber & Hedger, 1986).

6. If the aggressive subgroup is also present in the underlying xylem, then as the saprotrophic phase progresses colonisation of the bark by the aggressive will increase, by virtue of mutual synergism, even if the parent beetle sporeloads contained only a small proportion of aggressive spores.

7. The aggressive subgroup will be at an advantage because of its greater mycelial penetrating ability. This will lead to colonisation of increasing areas of bark at the expense of the non-aggressive subgroup. The enhanced sporulation associated with the penetration effect together with the lower fecundity of the non-aggressive subgroup (although not demonstrated for bark) will be likely to increase the number of aggressive subgroup spores available for secondary dispersal.

8. The greater area of bark colonised by the aggressive subgroup will lead to colonisation of a greater number of pupal chambers. If both subgroups are present in a pupal chamber, then the penetration effect and associated enhanced sporulation will increase the chances of aggressive isolates contributing to the sporeloads of emerging beetles.

9. The number of beetles carrying aggressive spores will continue to increase, and recycling of stages 4-9 will lead to replacement of the non-aggressive subgroup. The aggressive may be present for some years before increased disease levels become apparent, and there will be a further time lag before the aggressive can be detected in the *O.ulmi* population. The outcome of the battle between the aggressive and non-aggressive may well have been decided by the time the aggressive is detectable, and the decline of the non-aggressive very rapid from this point onwards.

The proposed model takes account of the factors identified in Chapter 8, and investigated in more detail in Chapter 9. The potential importance of these factors was predicted by Brasier (1986a),

but their function during the saprotrophic phases of both subgroups became more apparent from the work carried out Sections I and II. Other factors not discussed in the model are also likely to be involved in aggressive *versus* non-aggressive competition. For example, relative sexual and asexual fecundity, cytoplasmic infection, rate of asexual spread, and relative survival of spores on beetles during dispersal. Inter-subgroup competition will also take place in beetle feeding grooves, and recent work (Webber, 1987; unpublished data; Brasier, unpublished data) suggests that the penetration effect and relative growth rate, possibly influenced by pathogenicity and host resistance, will be important determining factors here too.

In the highly susceptible elm population North America, the two subgroups may be more evenly matched for growth rate in bark and in the colonisation of xylem. Other factors may be of greater importance, but differences between the subgroups are evidently still sufficient to lead to replacement of the non-aggressive. The increased understanding of factors leading to replacement of the non-aggressive subgroup resulting from this work will contribute to a wider understanding of the basic biology of Dutch elm disease. In the future, this may help achieve long term control of the disease by restoring the natural balance between pathogen and host.

Since planting of resistant elms and other control measures (Chapter 1.3) are not practical on a wide scale as a means to restore this balance, Brasier (1986a) has suggested direct genetic manipulation of the pathogen as an alternative. The interaction between the aggressive subgroup and any novel form of *O.ulmi* created via manipulation can in part be predicted from an understanding of the interaction between the aggressive and non-aggressive subgroups, since any new form would have to successfully compete with the aggressive. The possibility of hybridization between the new form and the aggressive would alter the range of possibilities, but assuming that selection would continue to favour a similar range of fitness attributes, then from the present observations it can be suggested that the new form of *O.ulmi* would require the following attributes in order to outcompete the aggressive:

1. The ability to move in the xylem without causing severe disease, and to feed back freely from the pathogenic phase to the saprotrophic phase.

2. The ability to colonise bark still exhibiting residual host

resistance (and possibly also to colonise feeding grooves) in competition with the aggressive subgroup.

3. Strong mycelial penetrating abilty, to invade and 'replace' mycelia of the aggressive subgroup.

4. Enhanced sporulation in association with the penetration effect to increase secondary dispersal in bark and sporulation in pupal chambers.

Some of these attributes will be closely coupled to strong pathogenic ability, and may therefore be incompatible with the objective of obtaining a better balance between pathogen and host via manipulation of the pathogen. Pathogenicity itself however, has many different components acting at different stages in the disease cycle. Successful infection of the xylem, mobility within the xylem, and the subsequent colonisation of dying bark, might each require a different range of genetic and biochemical attributes. Furthermore, the work presented here has continued to illustrate the complexity of the relationship between host, pathogen and vector, and emphasised that success for the pathogen does not depend on pathogenic ability alone. Strong penetrating ability, and high sexual and asexual fecundity, may be just as influential as pathogenicity in determining the outcome of competition, and might therefore be more useful objectives in any attempted manipulation of the host pathogen relationship.

REFERENCES

ADAMS, D.H. & ROTH, L.F. emarcation lines in paired cultures of *Fomes cajanderi* as a basis for detecting genetically distinct mycelia. *Canadian Journal of Botany* 45,1583-1589.

ANAGNOSTAKIS, S.L. (1982a). Genetic analysis of *Endothia parasitica:* linkage data for four single genes and three vegetative compatibility types. *Genetics* 102,25-28.

ANAGNOSTAKIS, S.L. (1982b). Biological control of chestnut blight. *Science* 215,466-471.

ANAGNOSTAKIS, S.L. (1983). Conversion to curative morphology in *Endothia parasitica* and its restriction by vegetative incompatibility. *Mycologia* 75,777-780.

BAKER, J.E. & NORRIS, D.M. (1968). Further biological and chemical aspects of host selection by *Scolytus multistriatus*. *Annals of the Entomological Society of America* 61,1248-1255.

BANFIELD, W.M. (1941). Distribution in the sap stream of spores of three fungi that induce vascular wilt diseases of elm. *Journal of Agricultural Research* 62,637-681.

BARRAS, S.J. (1972) Improved White's solution for surface sterilisation of pupae of *Dendroctonus frontalis*. *Journal of Economic Entomology* 65,1504.

BARRETT, D.K. & USCUPLIC, M. (1971). The field distribution of interacting strains of *Polyporus schweinitzii* and their origin. *New Phytologist* 70,589-598.

BAUGHMAN, M.J. (1985). Economics of Dutch elm disease control: A model and case study. *Journal of Forestry* 83,554-557.

BEATTIE, R.K. (1933). How the Dutch elm disease reached America. *National Shade Tree Conference* 9, 101-105.

BEAVER, R.A. (1966). The development and expression of population tables for the bark beetle *Scolytus scolytus F. Journal of Animal Ecology* 35,27-41.

BEAVER, R.A. (1967). Notes on the biology of the bark beetles attacking elm in Wytham Wood, Berks. *Entomologists Monthly Magazine* 102,156-162.

BEEVER, R.E. & BYRDE, R.J.W. (1982). Resistance to dicarboximide fungicides. In *Fungicide Resistance in Crop Protection* (ed. J. Dekker and S.G. Georgopoulos) pp. 101-117, Wageningen: Pudoc.

BECKMAN, C.H. (1956). Production of pectinase, cellulase and growth promoting substance by *Ceratocystis ulmi*. *Phytopathology* 46,605-609.

BERNIER, L., JENG, R.S. & HUBBES, M. (1983). Differentiation of aggressive and non-aggressive strains of *Ceratocystis ulmi by* polyacrylamide gel electrophoresis of intramycelial enzymes. *Mycotaxon* 17,456-472.

BETREM, J.G. (1929). De iepenziekte en de ipenspintkevers. *Tijdschrift over Plantenziekten* 35, 273-288.

BORDEN, J.H. & KING, C.J. (1977). Population aggregation pheromone produced by male *Scolytus scolytus* F. (Col.,Scolytidae). *Forestry Commission Research and Development Paper* 118.

BRASIER, C. (1977). Inheritance of pathogenicity and cultural characters in *Ceratocystis ulmi*; hybridization of protoperithecial and non-aggressive strains. *Transactions of the British Mycological Society* 68, 45-52.

BRASIER, C.M. (1978). Mites and reproduction in *Ceratocystis ulmi* and other fungi. *Transactions of the British Mycological Society* 70, 81-89.

BRASIER, C.M. (1979). Dual origin of recent Dutch elm disease outbreaks in Europe. *Nature, London* 281, 78-80.

BRASIER, C.M. (1981). Laboratory investigation of *Ceratocystis ulmi*. In *Compendium of Elm Diseases* (ed. R.J. Stipes & R.J. Campana) pp.76-79. St. Paul, Minnesota: American Phytopathological Society.

BRASIER, C.M. (1982). Occurrence of three sub-groups within *Ceratocystis ulmi*. In *Proceedings of the Dutch elm disease Symposium and Workshop*, Winnepeg, Manitoba, 1981. (ed. E.S. Kondo, Y. Hiratsuka & W.B.G. Denyer) pp.298-321. Manitoba Department of Natural Resources, Canada.

BRASIER, C.M. (1983a). The future of Dutch elm disease in Europe. *Forestry Commission Bulletin* 60, 96-104. HMSO, London.

BRASIER, C.M. (1983b). A cytoplasmically transmitted disease of *Ceratocystis ulmi*. *Nature, London* 305,220-223.

BRASIER, C.M. (1984). Intermycelial recognition systems in *Ceratocystis ulmi*: their physiological properties and ecological importance. In *The Ecology and Physiology of the Fungal Mycelium* (ed. D.H. Jennings & A.D.M. Rayner) pp.451-497. Cambridge University Press, London.

BRASIER, C.M. (1986a). The population biology of Dutch elm disease: its principal features and some implications for other host-pathogen systems. In *Advances in Plant Pathology* (ed. D.S. Ingram & P.H. Williams) 5,55-118. Academic Press, London and New York.

BRASIER, C.M. (1986b). A comparison of pathogenicity and cultural characteristics in the EAN and NAN aggressive sub-groups of *Ophiostoma ulmi*. *Transactions of the British Mycological Society* 87,1-13.

BRASIER, C.M. (1986c). The d-factor in *Ophiostoma ulmi*: its biological characteristics and implications for Dutch elm disease. In *Fungal Virology* (ed. K.W. Buck) pp.177-208. CRS Press, Boca Raton.

BRASIER, C.M. (1986d). Emergence of NAN/EAN hybrids of *Ophiostoma ulmi* in Europe. *Report on Forest Research* p37. HMSO, London.

BRASIER, C.M. (1987). The dynamics of fungal speciation. In *The Evolutionary Biology of the Fungi* (ed. A.D.M. Rayner, C.M. Brasier & D. Moore) pp.231-260. Cambridge University Press, London.

BRASIER, C.M. (1988). Rapid changes in genetic structure of epidemic populations of *Ophiostoma ulmi*. *Nature, London* 332,538-541.

BRASIER, C.M. & GIBBS, J.N. (1973). Origin of the Dutch elm disease epidemic in Britain. *Nature, London* 242, 607-609.

BRASIER, C.M. & GIBBS, J.N. (1975a). MBC tolerance in aggressive and non-aggressive isolates of *Ceratocystis ulmi*. *Annals of Applied Biology* 80, 231-235.

BRASIER, C.M. & GIBBS, J.N. (1975b). Highly fertile form of the aggressive strain of *Ceratocytis ulmi*. *Nature, London* 257,128-131.

BRASIER, C.M. & GIBBS, J.N. (1976). Inheritance of pathogenicity and cultural characters in *Ceratocystis ulmi*: hybridization of aggressive and non-aggressive strains. *Annals of Applied Biology* 83, 31-37.

BRASIER, C.M. & LEA, J. (1983). A fruiting succession in *Ceratocystis ulmi* and its role in Dutch elm disease. *Transactions of the British Mycological Society* 80,381-387.

BRASIER, C.M., LEA, J. & RAWLINGS, M.K. (1981). The aggressive and non-aggressive strains of *Ceratocystis ulmi* have different temperature optima for growth. *Transactions of the British Mycological Society* 76, 213-218.

BRASIER, C.M. & WEBBER, J.F. (1987). Positive correlations between *in vitro* growth rate and pathogenicity in *Ophiostoma ulmi*. *Plant Pathology* 36,462-466.

BRAYFORD, D (1983). *Phomopsis* as a biological control agent of Dutch elm disease. Ph.D. Thesis, University College of Wales, Aberystwyth

BUISMAN, C. (1932). Over het voorkomen van *Ceratostomella ulmi* (Schwarz) Buisman in de natur. *Tijdschrift over Plantenzeikten* 38,203-204.

BULLER, A.H.R. (1931). *Researches in Fungi* 4. Longmans Green, London.

BURDEKIN, D.A. & GIBBS, J.N. (1974). The control of Dutch elm disease. *Forestry Commission Leaflet* 54,7pp. HMSO, London.

BURDEKIN, D.A. & RUSHFORTH, K.D. (1981), revised by J.F. Webber, (1984). Breeding elms resistant to Dutch elm disease. 0.it on)Arboriculture Research Note (2 84 PATH) DOE Arboricultural Advisory and Information Service.

BURNETT, J.H. (1976). *Fundamentals of Mycology* 673 pp. Edward Arnold, London.

BURNETT, J.H. & PARTINGTON, D. (1957). Spatial distribution of fungal mating-type factors. *Proceedings of the Royal Philosophical Society of Edinburgh* 26,61-68.

CAMPBELL, A.H. (1938). Contribution to the biology of *Collybia radiata* (Reth) Berk. *Transactions of the British Mycologial Society* 22,151-159.

CATEN, C.E. (1972). Vegetative incompatibility and cytoplasmic infection in fungi. *Journal of General Microbiology* 72,221-229.

CATEN C.E. & JINKS, J.L. (1966). Heterokaryosis: Its significance in wild homothallic ascomycetes and fungi imperfecti. *Transactions of the British Mycological Society* 49,81-93.

CHAPMAN, J.W. (1910). The introduction of a European Scolytid, the smaller elm bark beetle, into Massachusetts. *Physche* 17,63-68.

CHILDS, T.W. (1963). *Poria weirii* root rot. *Phytopathology* 53,1124-1131.

COLLINS, C.W. (1941). Studies of elm insects associated with Dutch elm disease. *Journal of Economic Entomology* 34,369-372.

COLLINS, C.W., BUCHANAN, W.D., WITTEN, R.R. & HOFFMAN, C.H. (1936). Bark beetles and other possible insect vectors of the Dutch elm disease. *Journal of Economic Entomology* 29,169-176.

COOKE, R.J. & RAYNER, A.D.M. (1984). *Ecology of Saprotrophic Fungi*. Longmans, London.

CROFT, J.H. & DALES, R.B.G. (1984). Mycelial interactions and mitochondrial inheritance in *Aspergillus*. In *The Ecology and Physiology of the Fungal Mycelium* (ed. D.H. Jennings & A.D.M. Rayner) pp.433-450. Cambridge University Press, London.

CROFT, J.H. & JINKS, J.L. (1977). Aspects of the population genetics of *Aspergillus nidulans*. In *The Genetics and Physiology of* Aspergillus. (ed. J.E. Smith & J.A. Pateman) pp.339-360. Academic Press, London and New York.

ELGERSMA, D.M. (1970). Length and diameter of xylem vessels as factors in resistance of elms to *Ceratocystis ulmi*. *Netherlands Journal of Plant Pathology* 76,179-182.

ELGERSMA, D.M. (1973). Tylose formation in elms after inoculation with *Ceratocystis ulmi*, a possible resistance mechanism. *Netherlands Journal of Plant Pathology* 79,218-220.

ESSER, K. & BLAICH, R. (1973). Heterogenic incompatibility in plants and animals. *Advances in Genetics* 17,107-152.

FISHER, R.C. (1931). Notes on the biology of the large elm bark beetle, *Scolytus destructor* Ol. *Forestry* 5,120-131.

FISHER, R.C. (1937). The genus *Scolytus* in Great Britain, with notes on the structure of *Scolytus destructor* Ol. *Annals of Applied Biology* 24,110-130.

FRANSEN, J.J. (1931). Enkele gegevens omtrent de verspreiding van de door *Graphium ulmi* Schwarz veroorzaakte iepenziekte door de iepenspintkevers, *Eccoptogaster (Scolytus) scolytus* F. en *Eccoptogaster (Scolytus) multistriatus* Marsh in verband met de bestrijding dezer ziekte. *Tijdschrift over Plantenziekte* 37,49-62.

FRANSEN, J.J. (1939). Iepenziekte, iepenspintkevers en beider bestrijding. Dissertatie, Wageningen.

FRANSEN, J.J. & BUISMAN, C. (1935). Infectiproeven op verschillende iepensoorten met behulp van iepenspintkevers. *Tijdschrift over plantziekten* 41, 221-239.

GARNJOBST, L. (1953). Genetic control of heterokaryosis in *Neurospora crassa*. *American Journal of Botany* 40,607-614.

GARNJOBST, L. (1955). Further analysis of the genetic control of heterokaryosis in *Neurospora crassa*. *American Journal of Botany* 42,444-448.

GEORGOPOULOS, S.G. (1987). The development of fungicide resistance. In *Populations of Plant Pathogens* (ed. M.S. Wolfe & C.E. Caten) pp. 239-252. Blackwell.

GIBBS, J.N. (1978a). Development of the Dutch elm disease epidemic in southern England, 1971-6. *Annals of Applied Biology* 88, 219-228.

GIBBS, J.N. (1978b). Intercontinental epidemiology of Dutch elm disease. *Annual Review of Phytopathology* 16, 287-307.

GIBBS, J.N. & BRASIER, C.M. (1973). Correlation between cultural characters and pathogenicity in *Ceratocystis ulmi* from Britain, Europe and America. *Nature, London* 241, 381-383.

GIBBS, J.N. & BRASIER, C.M. (1980). Further studies on carbendazim tolerance in *Ceratocystis ulmi*. *Annals of Applied Biology* 94,273-310.

GIBBS, J.N. & CLIFFORD, D.R. (1974). Experiments with MBC derivatives for the control of Dutch elm disease. *Annals of Applied Biology* 78,309-318.

GIBBS, J.N. & GREIG, B.J.W. (1977). Some consequences of the 1975-1976 drought for Dutch elm disease in southern England. *Forestry* 50,145-154.

GIBBS, J.N. & WAINHOUSE, D. (1986). Spread of forest pests and diseases in the northern hemisphere. *Forestry* 59,141-153.

GIBBS, J.N., BRASIER, C.M., McNABB, H.S. & HEYBROEK, H.M. (1975). Further studies on pathogenicity in *Ceratocystis ulmi*. *European Journal of Forest Pathology* 5, 161-174.

GIBBS, J.N., HOUSTON, D.R. & SMALLEY, E.B. (1979). Agggressive and non-aggressive strains of *Ceratocystis ulmi* in North America. *Phytopathology* 69, 1215-1219.

GIBBS, J.N., LIESE, W. & PINON, J. (1984). Oak wilt for Europe? *Outlook on Agriculture* 13,203-207.

GREIG, B.J.W. (1982). Dutch elm disease - elm regeneration. *Report on Forest Research* pp. 25-26. HMSO, London.

GREIG, B.J.W. (1985). English elm regeneration. *Arboricultural Research Note* (13 85 PATH). DOE Arboricultural Advisory and Information Service.

GREIG, B.J.W. & GIBBS, J.N. (1983). Control of Dutch elm disease in Britain. *Forestry Commission Bulletin* 60,10-16. HMSO, London.

GUYOT, M. (1921). Notes de pathologie vegetale. *Bulletin de la Societe de Pathologie Vegetale de France* 8, 132-136.

HARRINGTON, T.C. (1981). Cycloheximide tolerance as a taxonomic character in *Ceratocystis*. *Mycologia* 73,1123-1129.

HANSEN, E.M., BRASIER, C.M., SHAW, D.S & HAMM, P.B. (1986). The taxonomic structure of *Phytophthora megasperma*: evidence for emerging biological species groups. *Transactions of the British Mycological Society*, 87, 557-573.

HEYBROEK, H.M. (1966). Dutch elm disease abroad. *American Forests* 72, 26-29 and 60-63.

HEYBROEK, H.M. (1976). Classification and nomenclature in the genus *Ulmus*. *Groen* 32,237-240.

HOLMES, F.W. (1958). Distribution of sexual compatibility strains of *Ceratocystis ulmi* in Massachusetts. *Phytopathology* 48,263.

HOLMES, F.W. (1962). Recorded Dutch elm disease distribution in North America as of 1961. *Plant Disease Reporter* 46, 715-718.

HOOG, G.S. de (1974). The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. *Studies in Mycology* 7, 1-84.

HOOG, G.S. de & SCHEFFER, R.J. (1984). *Ceratocystis versus Ophiostoma*: a reappraisal. *Mycologia* 76, 292-299.

HOUSTON, D.R. (1985). Spread and increase of *Ceratocystis ulmi* with cultural characteristics of the aggressive strain in northeastern North America. *Plant Disease* 69, 677-680.

HUBBES, M. (1975). Terpenes and unsaturated fatty acids trigger coremia formation by *Ceratocystis ulmi*. *European Journal of Forest Pathology* 5,129-137.

HUNT, J. (1956). Taxonomy of the genus *Ceratocystis*. *Lloydia* 19, 1-58.

IULI, R.J. & CAMPANA, R.J. (1985). Distribution and stability of aggressiveness in *Ceratocystis ulmi*. *Phytopathology* 75,626.

JENG, R.S. & HUBBES, M. (1983). Identification of aggressive and non-aggressive strains of Ceratocystis ulmi by poloyacrylamide gradient gel electrophoresis of intramycelial proteins. *Mycotaxon* 17,445-455.

JINKS, J.L. & GRINDLE, M. (1963). The genetical basis of heterokaryon incompatibility in *Aspergillus nidulans*. *Heredity* 18,407-413.

JINKS, J.L., CATEN, C.E., SIMCHEN, G. & CROFT, J.H. (1966). Heterokaryon incompatibility and variation in wild populations of *Aspergillus nidulans*. *Heredity* 21,227-239.

JOHNSON, R. (1987). Selected examples of relationships between pathogenicity in cereal rusts and resistance in their hosts. In *Populations of Plant Pathogens* (ed. M.S. Wolfe & C.E. Caten) pp. 181-192. Blackwell, Oxford.

KIRBY, S.G. & FAIRHURST, C.P. (1983). The ecology of elm bark beetles in northern Britain. *Forestry Commission Bulletin*(.bd on)60,47-49. HMSO, London.

KOSTICHKA, C.J. & CANNON, W.N. (1984). Costs of Dutch elm disease management in Wisconsin communities. *Journal of Arboriculture* 10,250-254.

LANE, E.B. (1981). Somatic incompatibility in fungi and Myxomycetes. In *The Fungal Nucleus* (ed. K. Gull & S.G. Oliver) pp.239-258. Cambridge University Press, London.

LEA,J. (1977). A comparison of the saprophytic and parasitic stages of *Ceratocystis ulmi*. Ph.D. Thesis, University of London.

LEA, J. & BRASIER, C.M. (1983). A fruiting succession in *Ceratocystis ulmi* and its role in Dutch elm disease. *Transactions of the British Mycological Society* 80,381-387.

LEROUX, P. & FRITZ, R. (1984). Antifungal activity of dicarboximides and aromatic hydrocarbons and resistance to these fungicides. In *Mode of Action of Antifungal Compounds* (ed. A.P.J. Trinci & J.F. Ryley) pp. 207-237, Cambridge University Press, London.

LIMING, F.G. (1934). A preliminary study of the lengths of the open vessels in the branches of American elm. *Ohio Journal of Science* 34,415-419.

MARCHAL, E. (1927). Rapport sur les resultats des recherches effectuees a la station de Phytopathologie de l'Etait a Gemloux sur la maladie l'orme. *Forestiere de Belgique* 35, 162-164.

MARSHALL, M.R., HINDAL, D.F. & MacDONALD, W.L. (1982). Production of perithecia in culture by *Ceratocystis ulmi*. *Mycologia* 74, 376-381.

MAZZONE, H.M. & PEACOCK, J.W. (1985). Prospects for control of Dutch elm disease- biological considerations. *Journal of Arboriculture* 11,285-292.

McNABB, H.S. (1974). Further speculation on the origin of the aggressive strain of *Ceratocystis ulmi*. *Abstract Paper 86th. Session of the Iowa Academy of Science, 1974* 12.

McNABB, H.S., HEYBROEK, H.M. & MacDONALD, W.L. (1970). Anatomical factors in resistance to Dutch elm disease. *Netherlands Journal of Plant Pathology* 76,196-204.

MELIN, E. & NANNFELDT, J.A. (1934). Researches into blueing of ground woodpulp. *Svenska Skogsford Forenings Tidsskrift* 32, 394-616.

MELVILLE, R. (1978). On the discrimination of species in hybrid swarms with special reference to *Ulmus* and the nomenclature of *U.minor Mill.* and *U.carpinifolia* Gled. *Taxon* 27,345-351.

MITCHELL, A.G. (1987). Characterization of iprodione tolerance in *Ophiostoma ulmi*. *Transactions of the British Mycological Society* 88,283-288.

MOORE, W.C. (1964). Progress in plant pathology. *Nature, London* 202,1041.

MOREAU, C. (1952). Coexistence des formes *Thielaviopsis* et *Graphium* chez une souche de *Ceratocystis* major (van Beyma) nov. comb. Remarques sur les variation des *Ceratocystis*. *Review Mycol.,17, Suppl. colon.*1, 17-25.

MOUNCE, I. (1929). Studies in forest pathology II. The biology of *Fomes pinicola* Fr. *American Journal of Botany* 12,163-177.

MYLYK, O.M. (1976). Heteromorphism for heterokaryon incompatibility genes in natural populations of *Neurospora crassa*. *Genetics* 83,275-284.

NEELY, D. (1973). Sanitation and Dutch elm disease control. In *Dutch Elm Disease* (ed. D.A. Burdekin & H.M. Heybroek). Proceedings of IUFRO conference, Minneapolis-St. Paul, USA. pp. 76-87. USDA Forest Service.

NEWBANKS, D. BOSCH, A. & ZIMMERMAN, M.H. (1983). Evidence for xylem disfunction by embolization in Dutch elm disease. *Phytopathology* 73,1060-1063.

O'CALLAGHAN, D.P. & FAIRHURST, C.P. (1983). Evaluation of the trap tree technique for the control of Dutch elm disease in northwest England. *Forestry Commission Bulletin* 60,23-28.

PARKER,K.G., READIO, P.A., TYLER, L.J. & COLLINS, D.L. (1941). Transmission of the Dutch elm disease pathogen by *Scolytus multistriatus* and the development of infection. *Phytopathology* 31,657-663.

PARMETER, J.R., SNYDER, W.C. & REICHLE, R.E. (1963). Heterokaryosis and variability in plant-pathogenic fungi. *Annual Review of Phytopathology* 1,51-76.

PARTRIDGE, A.D. & WEIR, L.C. (1974). Dutch elm disease moves into Oregon from Idaho. *Plant Disease Reporter* 58,75-76.

PEACE,T.R. (1960). The status and development of elm disease in Britain. *Forestry Commission Bulletin* 33. HMSO, London.

PEACE, T.R. (1962). *Pathology of Trees and Shrubs with special reference to Britain*. Oxford University Press, Oxford.

PEACOCK, J.W., LINCOLN, A.C., SIMEONE, J.B. & SILVERSTEIN, R.M. (1971). Attraction of *Scolytus multistriatus* (Coleoptera, Scolytidae) to a virgin female produced pheromone in the field. *Annals of the Entomological Society of America* 64,1143-1145.

POMERLEAU, R. (1961). History of Dutch elm disease in the Province of Quebec. *Forestry Chronicle* 37, 356-367.

PONTECORVO, G. (1956). The parasexual cycle in fungi. *Annual Review of Microbiology* 10,393-400.

PUHALLA, J.E. (1979). Classification of isolates of *Verticillium dahliae* based on heterokaryon incompatibility. *Phytopathology* 69,186-189.

PUHALLA, J.E. (1984a). A visual indicator of heterokaryosis in *Fusarium oxysporum from* celery. *Canadian Journal of Botany* 62,540-545.

PUHALLA, J.E. (1984b). Races of *Fusarium oxysporum* fsp *apii* in California and their genetic interrelationships. *Canadian Journal of Botany* 62,546-550.

PUHALLA, J.E. & HUMMEL, M. (1983). Vegetative compatibility groups within *Verticillium dahliae*. *Phytopathology* 73, 1305-1308.

PUHALLA, J.E. & SPEITH, P.T. (1983). Heterokaryosis in *Fusarium moniliforme*. *Experimental Mycology* 7,328-335.

PUHALLA, J.E. & SPEITH, P.T. (1985). A comparison of heterokaryosis and vegetative incompatibility among varieties of *Gibberella fujikuroi (Fusarium moniliforme)*. *Experimental Mycology* 9,39-47.

RAYNER, A.D.M. & TODD, N.K. (1979). Population and community structure and dynamics of fungi in decaying wood. *Advances in Botanical Research* 7,333-420.

RAYNER, A.D.M. & WEBBER, J.F. (1984). Interspecific mycelial interactions- an overview. In *The Ecology and Physiology of the Fungal Mycelium* (ed. D.H. Jennings & A.D.M. Rayner) pp.383-417. Cambridge University Press, London.

RAYNER, A.D.M., COATES, D., AINSWORTH, A.M., ADAMS, T.J.H., WILLIAMS, E.N.D. & TODD, N.K. (1984). The biological consequences of the individualistic mycelium. In *The Ecology and Physiology of the Fungal Mycelium* (ed. D.H. Jennings and A.D.M. Rayner) pp. 509-540. Cambridge University Press, London.

RICHENS, R.H. (1967). Studies on *Ulmus* VII. Essex elms. *Forestry* 40, 185-206.

RICHENS, R.H. (1977). New designations in *Ulmus minor* Mill. *Taxon* 26,,583-584.

RICHENS, R.H. (1980). On fine distinctions in *Ulmus* L. *Taxon* 29, 305-312.

RICHENS, R.H. (1983). *Elm*. Cambridge University Press, London.

RICHENS, R.H. & JEFFERS, J.N.R. (1978). Multivariate analysis of the elms of northern France II Pooled analysis of the elm populations of northern France and England. *Silvae Genetica* 27, 85-95.

RILEY, G.F. (1983). Dutch elm disease control campaign in Guernsey, Channel Islands, 1976-1981. *Forestry Commission Bulletin* 60,1-4.

ROGERS, H.J., BUCK, K.W. & BRASIER, C.M. (1986a). Transmission of double-stranded RNA and a disease factor in *Ophiostoma ulmi*. *Plant Pathology* 35, 277-287.

ROGERS, H.J., BUCK, K.W. & BRASIER, C.M. (1986b). The molecular nature of the d-factor in *Ceratocystis ulmi*. In *Fungal Virology* (ed K.W. Buck) pp 209-220. CRC Press, Florida.

SALEMINK, C.A., REBEL, H., KERLING, L.C.P. & TCHERNOFF, V. (1965). Phytotoxin isolated from liquid cultures of *Ceraocystis ulmi*. *Science* 149,202-203.

SCHEFFER, R.J. (1983). Biological control of Dutch elm disease by *Pseudomonas* species. *Forestry Commission Bulletin* 60,75-77.

SCHEFFER, R.J. & ELGERSMA, D.M. (1981). Detection of a phytotoxic glycopeptide produced by *Ophiostoma ulmi* in elm by enzyme-linked immunospecific assay (ELISA). *Physiological Plant Pathology* 18,27-32.

SCHEFFER, R.J. & ELGERSMA, D.M. (1982). A scanning electron microscope study of cell wall degradation in elm wood by aggressive and non-aggressive isolates of *Ophiostoma ulmi*. *European Journal of Forest Pathology* 12,25-28.

SCHNEIDER, I.R. (1956). A selective medium for the routine isolation of *Graphium ulmi* Schwarz. *Plant Disease Reporter* 40,816-821.

SCHWARZ, M.B. (1922). Das zweigensterben der omaen, trauerweiden und pfirschbaume. *Medelingen uit het Phytopathologisch laboratorium Willie Commelin Scholten*(,bd on) 5, 1-73.

SHAFER, T. & LIMING, O.N. (1950). *Ceratostomella ulmi* mating types in relation to the development and identification of perithecia. *Phytopathology* 40,1035-1042.

SHARLAND, P.R. & RAYNER, A.D.M (1986). Mycelial interactions in *Daldinia concentrica*. *Transactions of the British Mycological Society* 86,643-649.

SINCLAIR, W.A. (1978). Control stategies. In *Dutch elm disease: Perspectives after 60 years* p.30. Cornell University Agriculture Experiment Station *Search (Agriculture)* 8,30.

SKYLAKAKIS, S.K. (1987). Changes in the composition of pathogen populations caused by resistance to fungicides. In *Populations of Plant Pathogens* (ed. M.S. Wolfe & C.E. Caten) pp.227-238. Blackwell, London and New York.

SMUCKER, S.J. (1941). Comparison of susceptibility of the American elm and several exotic Asiatic elms to *Ceratostomella ulmi*. *Phytopathology* 31, 758-759.

SPIERENBERG, D. (1921). Een onbekende ziekte in de iepen. *Tijdschrift over Plantenziekten* 27, 53-60.

SVALDI, R. & ELGERSMA, D.M. (1982). Further studies on the activity of cell wall degrading enzymes of aggressive and non-aggressive isolates of *Ophiostoma ulmi*. *European Journal of Forest Pathology* 12,29-36

TAKAI, S. (1974). Pathogenicity and cerato-ulmin production in *Ceratocystis ulmi*. *Nature, London* 252,124-126.

TAKAI, S. (1980). Relationship of the production of the toxin, cerato-ulmin, to synnemata formation, pathogenicity, mycelial habit, and growth of *Ceratocystis ulmi* isolates. *Canadian Journal of Botany*, 58,658-662.

TAKAI, S. & HIRATSUKA, Y. (1984). Scanning electron microscope observations of internal symptoms of white elm following Ceratocystis ulmi infection and cerato-ulmin treament. Canadian Journal of Botany 62,1365-1371.

TAKAI, S. & RICHARDS, W.C. (1978). Cerato-ulmin, a wilting toxin of *Ceratocystis ulmi*: isolation and some properties of cerato-ulmin from the culture of *C.ulmi*. *Phytopathologische Zeitschrift* 91,129-146.

TAKAI, S., RICHARDS, W.C. & STEVENSON, K.J. (1983). Evidence for the involvement of cerato-ulmin, the *Ceratocystis ulmi* toxin, in the development of Dutch elm disease. *Physiological Plant Pathology* 23,275-280.

TCHERNOFF, V. (1965). Methods for screening and the rapid selection of elms for resistance to Dutch elm disease. *Acta Botanica Neerlandia* 14,409-452.

TODD, N.K. & RAYNER, A.D.M. (1980). Fungal individualism. *Science Progress, Oxford*. 66,331-354.

VanALFEN, N.K. & MacHARDY, W.E. (1978). Symptoms and host-pathogen interactions. *In Dutch elm disease: perspectives after 60 years* (ed. W.A. Sinclair & R.J. Campana). Cornell University Experimental Station *Search, Agriculture* 8,20-25.

VanALFEN, N.K. & TURNER, N.C. (1975). Influence of a *Ceratocystis ulmi* toxin on water relations of elm *Ulmus americana*. *Plant Physiology* 55,312-316.

VANDERPLANK, J.E. (1968). *Disease Resistance in Plants*. Academic Press, New York and London.

VERRAL, A.F. (1937). Variation in *Fomes ignarius*. *Minnesota Technical Bulletin* 117,1-41.

VERRALL, A.F. & GRAHAM, T.W. (1935). The transmission of *Ceratostomella ulmi* through root grafts. *Phytopathology* 25,1039-1040.

WALTER, J.M. (1937). Variation in mass isolates and monoconidium progenies of *Ceratostomella ulmi*. *Journal of Agricultural Research* 54,509-523.

WALTER, J.M. (1939). Observations on the fructification of *Ceratostomella ulmi* in England. *Phytopathology* 29,551-553.

WARCUP, J.H. (1955). On the origin of colonies developing on soil dilution plates. *Transactions of the British Mycological Society* 38, 298-301.

WATER, J.K. (1983). Dutch elm disease control in the Netherlands. *Forestry Commission Bulletin* 60,17-18.

WEBBER, J.F. (1979). Interactions between bark saprophytes and the Dutch elm disease pathogen *Ceratocystis ulmi*. Ph.D. Thesis, University College of Wales, Aberystwyth.

WEBBER, J.F. (1981). A natural biological control of Dutch elm disease. *Nature, London* 292,449-451.

WEBBER, J.F. (1983). Implications of fungicide tolerance. In *Report on Forest Research* p33. HMSO, London.

WEBBER, J.F. (1987). The influence of the d2 factor on survival and infection by the Dutch elm disease pathogen *Ophiostoma ulmi*. *Plant Pathology* 36,531-538.

WEBBER, J.F. & BRASIER, C.M. (1984). The transmission of Dutch elm disease: a study of the processes involved. *In Invertebrate-Microbial Interactions* (ed. A.D.M. Rayner & D.W.H. Walton) pp.271-306. Cambridge University Press, London.

WEBBER, J.F. & GIBBS, J.N. (1984). Colonization of elm bark by *Phomopsis oblonga*. *Transactions of the British Mycological Society* 82,348-352.

WEBBER, J.F. & HEDGER, J.N. (1986). Comparisons of interactions between *Ceratocystis ulmi* and elm bark saprobes in vitro and in vivo. *Transactions of the British Mycological Society* 86, 93-101.

WEBBER, J.F. & KIRBY, S.G. (1983). Host feeding preference of *Scolytus scolytus*. *Forestry Commission Bulletin* 60, 47-49.

WEBBER, J.F., BRASIER, C.M. & MITCHELL, A.G. (1987). The role of the saprophytic stage in Dutch elm disease. *In Plant Infecting Fungi* (ed. G.F. Pegg & P.G. Ayres) pp.298-313.

WEBBER, J.F., MITCHELL, A.G. & SMITH, F. (1986). Linkage of the genes determining mating type and fungicide tolerance in *Ophiostoma ulmi*. *Plant Pathology* 35,512-516.

WILSON, C.L. (1965). *Ceratocystis ulmi* in elm wood. *Phytopathology* 55,447.

WOLFE, M.S. (1984). Trying to understand and control powdery mildew. *Plant Pathology* 33,451-466.

WOLLENWEBER, H.W. (1928). Elm blight and its cause, *Graphium ulmi* Schwarz. *Bartlett Research Laboratories Bulletin* 1,26-31.

WOLLENWEBER, H.W. & STAPP, C. (1928). Untersuchungen uber die als Ulmensterben bekannte Baumkrankheit. *Arbeiten aus der biologischen Bundesanstalt fur Land-u Forstwirtschaft* 16, 282-324.

WOODS, A.C. & HOLMES, F.W. (1974). Extraction of fluid from healthy and Dutch elm diseased elm branches using hydaulic compression. *Phytopathology* 64,1265-1267.

YATES, M.G. (1984). The biology of the oak bark beetle, *Scolytus intricatus* Ratzeburg (Coleoptera: Scolytidae), in southern England. *Bulletin of Entomological Research* 74,569-579.

ZIMMERMAN, M.H. (1983). *Xylem Structure and the Ascent of Sap*. Springer-Verlag, Berlin.

APPENDIX 1 - PREPARATION OF MEDIA

2% OXOID MALT EXTRACT AGAR (MEA)

Oxoid malt extract agar	33 g
Oxoid technical agar	10 g
Distilled water	1000 ml

Autoclaved for 20 minutes at 121°C.

2% OXOID MALT EXTRACT AGAR WITH CYCLOHEXIMIDE AND STREPTOMYCIN (MEA+C+S) (Schneider, 1956, Brasier, 1981)

Members of the genus Ophiostoma are tolerant of cycloheximide, and the growth of other fungi can be conveniently suppressed by its inclusion in the isolation medium. Streptomycin was added to suppress bacteria.

As 2% MEA with:

Cycloheximide	0.10 g/l (50 ml of a 2 g/l stock solution)
Streptomycin	0.13 g/l (10 ml of a 13.33 g/l stock solution added after autoclaving)
Distilled water	940 ml

CARBENDAZIM SUPPLEMENT

A final carbendazim concentration of 0.5 µg/ml was obtained by including 5 ml of a 0.1 g/l stock solution in each 1000 ml of agar medium.

A 40 g/l stock solution was prepared with 40 g of technical grade carbendazim in 28 ml 1N hydrochloric acid and 472 ml of water. This was heated to ensure solution of the solid and diluted to 1000 ml with water. The 0.1 g/l stock solution was made from this by adding 2.5 ml to 992.5 ml of water and 5 ml of hydrochloric acid (Gibbs & Clifford, 1974).

IPRODIONE SUPPLEMENT

To achieve a final iprodione concentration of 5 µg/ml 5 ml of a 2 g/l stock solution of Rovral (Rhone Poulenc), containing 1 g/l iprodione, was added to each 1000 ml of autoclaved agar medium.

ELM SAPWOOD AGAR (ESA) (Brasier, 1981)

Elm sapwood	50 g
Oxoid technical agar	15 g
Distilled water	500 ml

Made up in a 1l bottle to prevent the medium boiling over, and autoclaved for 30 minutes at 121°C.

Elm sapwood was prepared from elm twigs approximately 10 mm in diameter. The bark was removed, the twigs cut into 30-50 mm lengths, oven dried at 100°C for 2 h or 80°C overnight and milled to fall through a 1-2 mm sieve.

ELM SAPWOOD AGAR FOR NON-AGGRESSIVE ISOLATES

Elm sapwood, prepared without removing bark	45 g
Linoleic acid	1 ml
Oxoid malt extract agar	1 g
Oxoid technical agar	15 g
Distilled water	500 ml

Autoclaved for 30 minutes at 121°C.

TCHERNOFF'S LIQUID MEDIUM (Tchernoff, 1965)

D-glucose	20 g
Asparagine	2 g
Potassium dihydrogen phosphate	1.5 g
Hydrated magnesium sulphate	1 g
Zinc sulphate	20 mg
Iron chloride	10 mg
Pyridoxine	1 mg
Thiamine	1 mg
Distilled water	1000 ml

Autoclaved for 20 minutes at 121°C.

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POPULATION STRUCTURES AND INTERACTION BETWEEN THE AGGRESSIVE AND NON-AGGRESSIVE SUBGROUPS OF *OPHIOSTOMA ULMI*

CORRECTIONS

Summary,	line 16	compatibility
Page 1,	line 8	acquired
	line 31	Gleditsch
	line 32	Salisb.
	line 35	Pall.
Page 4,	line 10	synnemata
Page 7,	line 19	Begin new paragraph at ´Evidence...´
Page 12,	line 1	artificial
	line 10	successful
	line 36	Begin new paragraph at ´Although...´
Page 16,	line 30	anastomosis
Page 19,	line 2	position
	line 4	incompatibility
Page 20,	line 2	*Fomes ignarius*
	line 16	explanation
	line 35	Anagnostakis, 1982a
Page 21,	line 15	Begin new paragraph at ´The widespread...´
	line 35	pressures
Page 23,	line 27	Begin new paragraph at ´The genetic...´
Page 24,	line 13	Anagnostakis, 1982a
Page 27,	line 9	investigation
Page 31,	line 7	discoloured
	line 16	availability
Page 37,	line 4	without
Page 41,	line 25	classes 2 and 3
Page 43,	line 20	ability
	line 21	synnemata
Page 48,	line 11	Begin new paragraph at ´A prerequisite...´
	line 16	identification
Page 49,	line 39	referred
Page 56,	line 30	occurring
Page 59,	line 59	demonstration
Page 60,	line 3	longitudinal
After	line 31	insert ´taken from near the bark/xylem interface, and the pattern of´
Page 62,	line 34	reactions
Page 63,	line 7	testing
Page 66,	line 5	they
Page 67,	line 18	Begin new paragraph at ´The method...´
Page 71,	line 14	Begin new paragraph at ´The population...´
	line 38	Furthermore
Page 75,	line 19	hierarchical
Page 78,	line 78	described
Page 79,	line 9	Begin new paragraph at ´The extent...´
Page 81,	line 19	*in vivo*
	line 30	ability
	line 33	described

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Page 82,	line 8	territorial
Page 84,	line 6	strengthened
Page 85,	line 3	compatibility
Page 86,	line 2	prerequisite
Page 88,	line 8	non-aggressive
Page 89,	line 27	phenomenon
Page 98,	line 12	populations
Page 99,	line 15	Begin new paragraph at 'The endemic...'
Page 105,	line 23	colonisation
Page 107,	line 22	phenomenon
Page 110,	line 13	phenomenon
Page 113,	line 9	adaptation
Page 114,	lines 23 and 36	pathogenicity
Page 119,	line 33	preceding
	line 37	streaking
	line 39	circumference
Page 120,	line 18	they
Page 121,	line 37	circumference
	line 29	Begin new paragraph at 'The results...'
Page 133,	line 17	significance
Page 134,	line 12	positions
Page 136,	line 6	interpretation
Page 140,	line 1	between
Page 142,	line 19	critical
Page 144,	line 14	assessments
Page 148,	lines 7 and 32	phenomenon
	line 10	between
Page 149,	line 4	Begin new paragraph at 'The results...'
Page 153,	line 5	preceding
Page 155,	line 20	hierarchical
Page 158,	line 18	beetles
	line 21	establishment
Page 161,	line 12	of North America
Page 162,	line 3	ability
Page 163,	line 1	(1967)
	line 1	Demarcation
Page 164,	line 47	*Ceratocystis*
Page 167,	line 12	Aggressive
Page 168,	line 11	polyacrylamide
Page 170,	After line 14 insert	'PUHALLA, J.E. (1985). Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Canadian Journal of Botany* **63**, 179-183.
Page 170,	lines 17 and 19	SPIETH
Page 170,	line 6	*Ceratocystis*
	line 27	strategies
Page 172,	line 3	treatment
Page 173,	line 31	hydraulic
Page 174,	line 9	*Ophiostoma*

A.G. MITCHELL, 1989